INTEGRATIVE ANALYSIS OF THE MUTATIONAL LANDSCAPE OF MOUSE AND HUMAN AML IDENTIFIES FUNCTIONALLY RELEVANT LEUKEMIA DISEASE ALLELES

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INTEGRATIVE ANALYSIS OF THE MUTATIONAL LANDSCAPE OF MOUSE AND HUMAN AML IDENTIFIES FUNCTIONALLY RELEVANT LEUKEMIA DISEASE ALLELES

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t(8;21) is one of the most frequent chromosomal abnormalities observed in patients with acute myeloid leukemia (AML). However, expression of AML1-ETO is not sufficient to induce transformation *in vivo*. Consistent with this observation, patients with this translocation harbor additional genetic abnormalities, suggesting a requirement for cooperating mutations. To better define the genetic landscape in AML and distinguish driver from passenger mutations, we compared the mutational profiles of AML1-ETO driven mouse models of leukemia to the mutational profiles of human AML patients. We identified TET2 and PTPN11 mutations in both mouse and human AML and then demonstrated the ability of *Tet2* loss and PTPN11 D61Y to initiate leukemogenesis in concert with expression of AML1-ETO *in vivo*. This integrative genetic profiling approach allowed us to accurately predict cooperating events in t(8;21)+ AML in a robust and unbiased manner, while also revealing functional convergence in mouse and human AML.

BIOGRAPHICAL SKETCH

Education

Cornell University - Weill Cornell Graduate School of Medical Sciences
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Personal Statement

Megan Hatlen is a PhD candidate in Cellular Biology at the Weill Cornell Graduate School of Medical Sciences of Cornell University and possesses over 10 years of experience in the fields of cellular and molecular biology. Her primary research interest is oncology, specifically, hematology/oncology with a focus on acute myeloid leukemia. This interest has been highlighted by research performed during her PhD candidacy in the laboratories of Dr. Stephen Nimer and Dr. Ross Levine at Memorial Sloan Kettering Cancer Center. While in these laboratories, she developed an integrated genomic profiling approach that identified disease alleles capable of cooperating with AML1-ETO to initiate leukemia. She then validated several of these disease alleles by demonstrating that their expression in AML1-ETO positive cells was sufficient to initiate

leukemia *in vivo*. Through this work, Megan has become proficient at modeling oncogenesis and assessing therapeutic efficacy,

Her expertise in molecular biology is highlighted by work performed while obtaining her Bachelors of Science in Bioengineering: Biotechnology at the University of California at San Diego. During this time, Megan carried out research in the laboratory of Dr. Joseph Ecker at the Salk Institute for Biological Studies. She utilized a forward genetics screen in *Arabidopsis thaliana* to identify regulatory factors of the exoribonuclease EIN5. Once identified, she worked with a team to characterize the function of the regulatory factors, as well as their impact on microRNA expression and ethylene signal transduction.

During these and other studies, Megan has had the opportunity to initiate and maintain collaborations with laboratories in similar fields, as well as the nonprofit institution, the New York Genome Center. She has also carried out independent work while managing and mentoring research technicians. These skills and the expertise that she has developed make her uniquely qualified to contribute to the fields of hematology and oncology.

Research Experience

Memorial Sloan Kettering Cancer Center 2007 - Present
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PhD Candidate

The translocation between chromosomes 8 and 21 creates a fusion protein known as AML1-ETO, which has been proven insufficient for leukemogenesis. The goal of this study was to identify disease alleles capable of cooperating with AML1-ETO to initiate leukemia *in vivo* using the genomics of both mouse and human AML. In seeking to identify cooperating alleles, Megan generated two novel mouse models of t(8;21) driven Acute Myeloid Leukemia. These models provide information on the requirements for AML1-ETO leukemogenesis, and can be used to determine if targeted therapeutics against either disease allele would be beneficial.

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The goal of this study was to identify factors that were involved in ethylene signal transduction through their regulation of the exoribonuclease EIN5. Potential factors were identified via a forward genetic screen in which EIN5 mutant plants were given random additional genetic mutations through ethyl-methanesulfonate (EMS) mutagenesis. EMS treated plants that showed an enhancement in their original EIN5 mutant phenotype were selected, and subsequent outcrossing to a wildtype plant revealed whether the phenotype of the secondary mutations were dependent upon the original EIN5 mutation. The role of factors which regulate EIN5 were then investigated and found to control microRNA biogenesis. The spatial and temporal changes in micoRNA expression were assessed via qPCR and Nothern blotting.

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The goal of this study was to generate IgE peptides that could be used to downregulate the IgE response through immunization. After injecting mice with various peptides, cytotoxic T-cell activity was assessed using ^{51}Chromium Release Assays. Cytotoxic T-cell activity was also determined by measuring IFNγ secretion using ELISA or ELISPOT assays.

Publications

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This doctoral dissertation is dedicated to all members of my loving family.

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In my brother, Tyler Hatlen, I have always found a fount of understanding and comradeship. It comforts me to know I am guaranteed a reflection, a partner, a friend.

To my wife, Jessica Hatlen: Without you, nothing is possible. With you, everything.

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INTRODUCTION

Hematopoiesis

Hematopoiesis is a complex process by which an immature cell, capable of generating identical daughter cells, gives rise to mature progeny each capable of executing a specific function within the blood and bone marrow compartment. The hierarchical nature of this process was first postulated by biologist A. Maximow[1], but could not be substantiated until later studies pioneered the use of bone marrow transplantation. During the atomic era, transplantation of bone marrow or spleen cells from a healthy individual into one exposed to radiation was found to preclude lethal bone marrow failure[2]. This finding supported the existence of cells capable of reconstituting the hematopoietic system but could not determine whether mature cells originated from a single immature cell or multiple immature cells, each responsible for a specific blood lineage. Seminal studies in mice which demonstrated that multiple blood lineages could be reconstituted from the cells that formed macroscopic colonies in the spleen after transplantation provided the evidence required for attributing all mature cells to a single immature cell[3,4], referred to as a hematopoietic stem cell. Scientists have since refined the morphological features and cell surface proteins that characterize stem cells, subsequent progenitor cells, and various mature cells, organizing the heterogeneous blood and bone marrow compartment into one in which cellular identity and potential is defined.

It is understandable that a complex process of cellular maturation be regulated in a similarly complex manner. However, the process of maturation, known as differentiation, can best be described at the cellular level for a hematopoietic stem or

progenitor cell as a decision to generate either two identical daughter cells, an identical daughter cell and a more differentiated daughter cell, or two differentiated daughter cells. This cell fate decision has been described in probabilistic terms[5], and has largely been attributed to changes in the expression of a variety of genes at specific time points. Changes in gene expression, in turn, are regulated by a plethora of factors, including the expression of transcription factors[6], epigenetic factors[7,8], RNA machinery[9], and the initiation of cytokine signaling cascades[10].

One gene critical to hematopoiesis is AML1 (also known as RUNX1). AML1 itself is a transcription factor that, owing to alternative splicing, can possess varying amounts of C-terminal transcript and, as a result of alternative promoter usage, can express an extended N-terminal transcript[11,12]. Conserved in the majority of AML1 isoforms is a runt homology domain that allows AML1 to bind DNA at a specific consensus sequence[13] and regulate the expression of genes relevant to differentiation. It is important to note that while AML1 has been found to activate the expression of cytokines such as IL3[14] and GM-CSF[15], and factors important to granulopoiesis, such as myeloperoxidase[16], MCSF receptor[17], and neutrophil elastase[18], AML1 itself is a weak activator and typically achieves gene regulation via the recruitment of different co-activators, including MEF[19], C/EBPα, PU.1[17], CBP/p300[20], and c-MYB[21], to different target genes. In this manner, AML1 is capable of acting broadly, altering the expression of a wide variety of hematopoietic genes, gene regulators, and signaling pathways. Mice null for AML1 die at an early embryonic stage, experiencing a complete block in definitive hematopoiesis. Only yolk-sac derived primitive hematopoiesis remains intact[22,23]. The fact that a single transcription factor could have such a lethal effect underscores the importance not only of hematopoiesis, but of its regulation.

Acute Myeloid Leukemia

The loss, disruption, or mutation of genes relevant to hematopoiesis can have lethal effects, and that lethality at times results from the development of leukemia. Leukemia is defined as the clonal expansion of immature hematopoietic cells with impaired differentiation potential. Accordingly, two biological features have become hallmarks of a leukemic state: continuous proliferation and concurrent differentiation arrest[24].

Acute myeloid leukemia (AML) is subtype of leukemia in which the malignant stem or progenitor cell is one that would not have contributed to the development of lymphocytic cells, but instead would have contributed to the development of cells belonging to the myeloid lineage. The annual incidence of AML is approximately 2.4 in 100,000 in the United States[25], and this rate increases more than 5 fold for individuals older than 65. Despite having a lower incidence rate, individuals younger than 65 still face a survival rate of only 40%[26]. The majority of AML patients present with cytogenetic abnormalities[27,28], and their resulting karyotype is an independent predictor of overall survival. Of note, balanced chromosomal translocations are common in AML, and the translocation breakpoint occurs at genes that are almost always transcription factors. In several cases, a chimeric fusion protein is generated via the expression of two different genes at either side of the breakpoint. This fusion protein can interfere with the functions of either affected gene or assume novel functions itself[24]. The predominance of chromosomal aberrations in AML and their impact on patient survival has prompted investigations into the mechanisms by which the affected genes may contribute to malignant cell proliferation or differentiation arrest.

Two-Hit Hypothesis

While a hallmark of acute myeloid leukemia is the targeting of transcription factors by chromosomal aberrations, the chromosomal translocations present in chronic myeloid leukemia (CML) overwhelmingly target tyrosine kinases. In the majority of these translocations, a fusion protein is generated that harbors not only a tyrosine kinase domain, but an oligomerization motif which facilitates the constitutive activation of the respective kinase. When expressed in hematopoietic cell lines, the fusion protein is capable of activating cytokine signaling cascades such that cellular growth is possible in the absence of cytokines. When expressed in animal models, the fusion protein consistently induces a myeloproliferative neoplasm (MPN), a disorder characterized by the accumulation of a single mature cell type[29].

The expression of transcription factor fusion proteins in AML does not promote cytokine independent cell growth; instead, the primary consequence is an impairment in differentiation. This impairment in part can result from the loss-of-function of one or both genes involved in the translocation. For example, expression of the t(8;21) fusion product, AML1-ETO, results in embryonic lethality and a block in definitive hematopoiesis[30,31], a phenotype reminiscent of AML1 loss. Notably, transcription factor fusion proteins present in AML rarely induce overt leukemia when expressed alone. Conditional expression of AML1-ETO in murine models is capable of inducing leukemia only after mutagenic agents like *N*-ethyl-*N*-nitrosourea have been employed[32,33]. Fusion proteins such as CBFβ-SMMHC and PML-RARα, induce leukemia with incomplete penetrance after an extensive latency[34,35]. These results suggest a requirement for secondary genetic events in leukemias characterized by transcription factor fusion proteins.

Such a requirement has been substantiated by studies of CML and MPNs and their progression to AML. In a select number of cases, a secondary genetic event not identified in the initial diagnosis is discovered in the resultant AML. For instance, patients with BCR-ABL positive CML have been found to acquire t(7;11), a translocation which results in expression of the NUP98-HOXA9 fusion protein, during blast crisis[36,37]. Similarly, a patient presenting with the MPN known as Polycythemia vera, and harboring the JAK2 V617F mutation has been found to progress to AML after acquiring AML1-ETO[38]. These observations not only support the co-occurrence of secondary events with transcription factor fusion proteins, but also suggest a model of AML that is reliant upon the acquisition of two classes of mutations. The first class of mutations (Class I) is typified by those present in CML and MPNs; the mutations confer a proliferative advantage to cells, without affecting their ability to differentiate. The second class of mutations (Class II) is typified by transcription factor fusion proteins, which impair differentiation but do not provide a proliferative advantage[29]. A cell that has acquired mutations in both classes is capable of the clonal outgrowth which defines AML. This model of leukemogenesis is now known as the two-hit hypothesis.

AML1-ETO*

AML1-ETO is an exemplary Class II mutation. And while the translocation between chromosomes 8 and 21 was first identified in AML by Dr. Janet Rowley more than four decades ago[39], investigators have only recently begun to understand the functions of AML1-ETO and its role in leukemogenesis. Clinically, the t(8;21) translocation

* Hatlen MA and Nimer SD. AML1-ETO driven acute leukemia: insights into pathogenesis and potential therapeutic approaches. *Frontiers of Medicine*. **6**, 248-262 (2012).

represents the most frequent chromosomal abnormality in AML, occurring in approximately 4%–12% of adult and 12%–30% of pediatric patients[40]. t(8;21)+ leukemias exhibit some degree of myeloid differentiation, which has led to their classification as a M2 subtype of AML, based on the French-American-British (FAB) Working Group Classification system[41,42]. Several groups identified the AML1 gene on chromosome 21 as being fused to the ETO gene on chromosome 8, and as a result of this work the AML1-ETO fusion cDNA was ultimately cloned. AML1-ETO contains the RUNT homology domain of AML1 and nearly the full length open reading frame of the ETO gene[43,44,45,46,47,48].

While the N-terminal component of the AML1-ETO fusion protein, AML1, is a transcription factor that regulates the expression of a variety of genes related to myeloid and lymphoid differentiation, the role of the C-terminal component of AML1-ETO, ETO (also known as MTG8), in hematopoiesis is less well defined. There is no expression of murine ETO in early mouse hematopoietic cells, and mice lacking ETO have no obvious defects in hematopoiesis; their primary phenotype is a reduction in postnatal viability and impaired midgut formation[49]. It is generally believed that ETO and its family members ETO-2/MTG16 and MTGR1 act in transcriptional silencing, as they bind corepressors such as nuclear receptor corepressor (NCoR), silencing mediator for retinoid/thyroid hormone receptors (SMRT), mSin3a, and various members of the histone deacetylase (HDAC) family via four evolutionarily conserved nervy homology regions (NHR)[50,51,52]. These domains are retained in the AML1-ETO fusion protein, along with the DNA binding domain of AML1. Initial studies of AML1-ETO identified it as a dominant inhibitor of AML1 function[53,54], and were supported by the knock-in mouse model of AML1-ETO, which largely replicates the

phenotype of AML1 null mice (Table 1). However, while the AML1 null fetal liver lacks hematopoietic stem/progenitor cells (HSPCs), the fetal livers of AML1-ETO

Table 1. Mouse Models of AML1-ETO Expression

Model	Phenotype
Transgenic models	
AML1-ETO knocked-in to the Cbfα2 locus	- Mice died during embryogenesis, 12.5–13.5 days post coitum - Mice showed hemorrhaging in the central nervous system - Fetal liver hematopoiesis was blocked
AML1-ETO knocked-in to the Cbfα2 locus	- Mice died during embryogenesis, 12.5–14.5 days post coitum - Mice showed hemorrhaging in the central nervous system - Fetal liver hematopoiesis was blocked
Mice expressing AML1-ETO cDNA under a tetracycline-responsive promoter were bred to mice containing the murine mammary tumor virus-tet controlled transcriptional activator (MMTV-tTA) construct	- Mice showed normal hematopoiesis - Bone marrow cells showed enhanced serial replating ability
Mice in which a chromosomal translocation between AML1 and ETO genes could be generated through Cre/loxP-mediated recombination were crossed to Nestin-Cre mice	- The translocation was not detectable by PCR in DNA isolated from bone marrow, muscle, and lung. Trace amounts of the translocation were found in DNA isolated from testes, spleen, and liver - No malignancies were detected in mice aged 5 months or younger
Mice expressing AML1-ETO cDNA under the human MRP8 promoter	- Mice showed normal hematopoiesis - Treatment of newborn hMRP8-AML1-ETO transgenic mice with N-ethyl-N-nitrosourea resulted in 55% developing AML
Mice with a conditional AML1-ETO knock-in allele (under the endogenous AML1 promoter) were crossed to Mx1-Cre mice	- Mice showed normal hematopoiesis - Bone marrow cells showed enhanced serial replating ability - Treatment with N-ethyl-N-nitrosourea resulted in 31% developing granulocytic sarcoma/AML
Mice contained an AML1-ETO-IRES-eGFP construct under the Ly6A locus	- Mice developed a spontaneous myeloproliferative disorder with a penetrance of 52% at 1 year and 82% at 14 months.
Transplantation models	
Mouse c-Kit$^+$Sca-1$^+$Lin$^-$ bone marrow cells were transduced with the MSCV-AML1-ETO-IRES-eGFP cDNA construct and transplanted into lethally irradiated congenic recipients	- Mice showed an increased number of myeloid progenitors and c-Kit$^+$Sca-1$^+$Lin$^-$ cells in the bone marrow compartment - No hematopoietic malignancies were detected
Mouse whole bone marrow or whole E14.5 fetal liver cells were transduced with the truncated AML1-ETO cDNA (Mig-A/Etr) construct and transplanted into lethally irradiated recipients. MF-1, BALB c, or C57 mice were used	- Mice developed acute myeloid leukemia - MF-1 mice transplanted with transduced MF-1 E14.5 fetal liver cells had a median survival time of 20 weeks post-transplantation - MF-1 eGFP$^+$ splenocytes stained as Lin$^-$, Sca-1$^-$, c-Kit$^+$, FcyRII/III^{med}, and CD34$^-$
MF-1 mouse whole E14.5 fetal liver cells were transduced with the AML1-ETO9a cDNA (Migr1-AE9a) construct and transplanted into lethally irradiated MF-1 recipients	- The majority of the mice developed acute myeloid leukemia 16 weeks post-transplantation - eGFP$^+$ cells stained as Lin$^-$, Sca-1$^-$, c-Kit$^+$, FcyRII/III^{med}, and CD34$^-$
C57BL/6 mouse whole E14.5 fetal liver cells were transduced with the AML1-ETO9a cDNA (Migr1-AE9a) construct and transplanted into lethally irradiated C57BL/6 recipients	- Mice developed acute myeloid leukemia and had a median survival time of 25 weeks post-transplantation - eGFP$^+$ cells stained as Lin$^-$, Sca-1$^-$, c-Kit$^+$, FcyRII/III^{med}, and CD34$^-$
Whole E14.5 fetal liver cells from MRP8-AE transgenic mice were transduced with the AML1-ETO9a cDNA (Migr1-AE9a) construct and transplanted into lethally irradiated C57BL/6 recipients	- The majority of the mice developed acute myeloid leukemia 5 weeks post-transplantation - The eGFP$^+$ Lin$^-$Sca-1$^-$c-Kit$^+$, as well as the eGFP$^+$ Lin$^-$Sca-1$^+$ c-Kit$^+$ cells, were FcyRII/III^{lo-hi} and CD34$^+$
Human CD34$^+$ hematopoietic cells were transduced with the MigR1-AML1-ETO cDNA construct and transplanted into NOD/SCID mice	- AML1-ETO expressing CD34$^+$ cells had an enhanced self-renewal and proliferative ability - Engraftment in NOD/SCID mice was limited and did not result in leukemogenesis
Human CD34$^+$ hematopoietic cells were transduced with FMEV retrovirus containing the AML1-ETO cDNA construct and transplanted into NOD/SCID mice	- AML1-ETO expressing CD34$^+$ cells had an enhanced self-renewal ability - Engraftment in NOD/SCID mice was achieved, but did not result in leukemogenesis

knock-in mice contain small numbers of multilineage progenitor cells with dysplastic morphology, which possess increased self-renewal capacity when cultured in vitro[30,31]. These results allude to functions of AML1-ETO that are distinct from its ability to block AML1 function, and that are likely also critical to leukemogenesis.

Transgenic Expression of AML1-ETO

Efforts to establish mouse models of t(8;21)+ AML, and bypass the embryonic lethality of AML1-ETO "knock-in" expression, led to the generation of several mouse models where AML1-ETO was conditionally expressed (Table 1). An elegant model, in which the translocation between the murine AML1 and ETO genes was induced via CRE/LoxP mediated recombination, showed no leukemia when CRE was expressed from the Nestin promoter. However, the use of the Nestin promoter to induce recombination in this model was not ideal, as it generated the translocation in brain, kidney, and heart tissues, but not in bone marrow cells[55]. The Zhang laboratory subsequently generated a transgenic mouse in which the expression of AML1-ETO was under the control of a tetracycline-responsive element. In the absence of tetracycline, the AML1-ETO protein was detectably expressed in bone marrow cells, and although the animals did not develop leukemia, the bone marrow cells expressing AML1-ETO did show increased self-renewal and a partial block in myeloid differentiation[33].

A similar phenotype was seen with a conditional AML1-ETO knock-in mouse model. By placing a transcriptional stop cassette flanked by LoxP sites in the third intron of AML1, and inserting the remaining AML1-ETO cDNA in frame to exon 4, the Downing laboratory created an AML1-ETO allele that could be expressed from the endogenous AML1 promoter following CRE/LoxP mediated recombination[32]. When

these mice were crossed with *Mx1-Cre*$^{+/-}$ mice[56], and poly(I:C) treatment was used to delete the stop cassette, AML1-ETO expression occurred in the hematopoietic compartment. AML1-ETO increased the self renewal of the bone marrow cells without impairing their ability to form colonies in methylcellulose cultures, although there was a slight skewing toward the granulocyte/macrophage lineage. Importantly, AML1-ETO expression did not initiate leukemia in this knock-in model[32].

To induce acute leukemia in this conditional knock-in model, the *AML1-ETO-Stop/+; Mx1-Cre*$^{+/-}$ mice were treated first with poly(I:C) and then *N*-ethyl-*N*-nitrosourea (ENU) to generate additional genetic events. Nearly half of these mice developed hematopoietic neoplasms, which were largely granulocytic sarcoma/acute myeloid leukemia, with features reminiscent of t(8;21)+ AML. The remaining neoplasms were thymic-derived T cell lymphoblastic lymphomas that did not express AML1-ETO[32]. A similar result was seen when ENU was administered to transgenic mice in which AML1-ETO was expressed under the human MRP8 promoter; approximately half of the treated mice succumbed to AML while the remaining mice succumbed to lymphoma or acute lymphocytic leukemia (ALL). These MRP8-AML1-ETO transgenic mice did not develop disease without ENU treatment[57], and the occurrence of lymphoma or ALL in the ENU treated mice is not surprising, as ENU induces lymphomas or ALL in wildtype mice[58].

Taken together, these models clearly show that AML1-ETO expression alone is insufficient for leukemia initiation. Ultimately, the increase in self-renewal that AML1-ETO provides must be supplemented with additional genetic or epigenetic events to initiate leukemia. This is compatible with the in utero detection of the t(8;21) AML1-ETO translocation in children who develop AML later in life, even

more than 10 years after birth[59]. It is also supported by the detection of additional genetic abnormalities in t(8;21)+ AML patients at diagnosis, such as the loss of a sex chromosome, chromosomal deletions or trisomies, and mutations in receptor tyrosine kinases[60]. In fact, although they did not report on AML1-ETO positive AML specifically, data emerging from the whole genome sequencing efforts of the Washington University group on primary human AML patient samples have demonstrated that the "average" AML likely contains dozens of mutations[61,62].

Mouse Models of AML1-ETO Driven Leukemogenesis

To better understand which secondary events can cooperate with AML1-ETO to induce leukemia, investigators have taken advantage of retroviral transduction and transplantation experiments in mice, introducing AML1-ETO together with a variety of cDNAs. When bone marrow Lin-Sca-1$^+$c-Kit$^+$ (LSK) cells, a population highly enriched for HSPCs, are transduced with AML1-ETO expressing retrovirus alone, and transplanted into lethally irradiated congenic recipient mice, the mice do not develop leukemia. Instead, AML1-ETO positive myeloblasts are found in the bone marrow of recipient mice 10 months post-transplantation, and these cells, which may represent 10% of the bone marrow population, show increased self-renewal in colony forming assays[63]. However, when bone marrow cells are transduced with AML1-ETO and either the TEL-PDGFβR fusion protein[64], the FLT3 length mutation (FLT3-LM)[65], or the c-KIT N822K mutation[66], recipient mice succumb to fatal AML with a median latency of 60, 233, or 178 days, respectively. Of these secondary events, the c-KIT mutation is the most commonly identified mutation in t(8;21)+ leukemia patients. At euthanasia, the leukemic mice in each of these transplant models show leukocytosis, anemia, thrombocytopenia, splenomegaly, and a blast population that exceeds 20% of the bone marrow cells. These leukemias require the DNA binding ability of AML1-

ETO[64,65,66] and they are transplantable into sub-lethally irradiated recipient mice. For instance, when TEL-PDGFβR is paired with an AML1-ETO L148D mutant that lacks the ability to bind DNA (or interact with CBFβ), the recipient mice develop a MPN similar to that seen from TEL-PDGFβR expression alone[64]. FLT3-LM and c-KIT N822K also give rise to a MPN when expressed individually[67].

Because these mutations result in the constitutive activation of a tyrosine kinase that confers a proliferative advantage to hematopoietic cells, they are each considered a Class I mutation[68,69]. The ability of AML1-ETO to cooperate with three different Class I mutations lends validity to the two-hit hypothesis; however, as more mutations are identified in AML patients, events capable of cooperating with AML1-ETO have been discovered that do not necessarily comply with the requirements of a Class I mutation. This suggests that while the two-hit hypothesis is clearly biologically applicable to AML, as mutations of a given class generally do not co-occur in patients, it may represent an oversimplification of AML.

One event that does not fulfill the tenets of a Class I mutation, yet cooperates with AML1-ETO, is the overexpression of transcription factor WT1. WT1 is upregulated in AML patients[70], and although originally described as a tumor suppressor gene, the overexpression of WT1 has been found to not only promote the proliferation of mouse bone marrow progenitor cells, but to also inhibit their differentiation in response to G-CSF[71]. Mice transplanted with bone marrow cells from WT1 overexpressing transgenic (WT1-Tg) mice have splenomegaly and an increased number of mature myeloid cells and myeloid progenitor cells in the bone marrow; however, they do not develop AML. When AML1-ETO is expressed in WT1-Tg bone marrow cells, the recipient mice die of an aggressive, invasive AML 1 to 4 months post-transplant[72].

In a similar scenario, AML1-ETO cooperates with the loss of interferon consensus sequence binding protein (ICSBP, also known as interferon regulatory factor 8) to induce myeloblast transformation in mice. ICSBP expression is downregulated in AML patient samples, which prompted experiments expressing AML1-ETO in ICSBP$^{-/-}$ mouse bone marrow cells. While the transplantation of ICSBP$^{-/-}$ bone marrow cells leads to a MPN, the expression of AML1-ETO in these cells leads to an accumulation of myeloblasts in the bone marrow of the recipient mice and non-fatal granulocytic sarcomas[73]. Though not AML, the development of this latter pathological feature, which is present in some t(8;21)+ AML patients[74], is suggestive of ICSBP loss playing an important role in disease progression to overt AML.

It may in fact be possible for AML to result from an accumulation of multiple functional hits, just as it is possible for AML to result from the acquisition of two classical hits. Through studies in myeloid cell lines, the Zhang laboratory found that AML1-ETO could trigger growth arrest by binding to the p21 promoter and inducing its expression[75]. They hypothesized that growth arrest and p21 upregulation must be circumvented for AML1-ETO to be fully leukemogenic, possibly through the actions of a cooperating hit. Accordingly, when AML1-ETO was expressed in p21$^{-/-}$ mouse fetal liver cells, many of the recipient mice developed a lethal AML after a latency of at least 4 months. The extensive latency, the fact that the p21 gene is rarely inactivated in patients with AML[76,77,78,79], and the fact that t(8;21)+ AML patient samples generally overexpress p21[80], makes it unlikely that the loss of p21 directly synergized with AML1-ETO expression in this mouse model of AML. Instead, this model appears to demonstrate the importance of certain p21 regulated processes in AML1-ETO driven leukemogenesis. p21 plays a role in DNA damage repair[81,82,83], and its

loss could result in the accumulation of mutations and/or epigenetic events that contribute to leukemogenesis in the presence of AML1-ETO. In this scenario, at least two genetic events, p21 loss and an acquired mutation, cooperate with AML1-ETO.

Aside from cooperativity studies, our understanding of the leukemogenic potential of AML1-ETO has been significantly advanced by the identification of an AML1-ETO splice variant, AML1-ETO9a, which can induce AML after a 5-6 month latency. In 2004, the Zhang laboratory transplanted mouse bone marrow cells that had been retrovirally transduced with AML1-ETO into recipient mice. One of the recipient mice developed AML 14 weeks post-transplantation without any artificially induced mutations. Sequencing of the AML1-ETO cDNA in the leukemic cells revealed a single base-pair insertion that resulted in a C-terminal truncation of approximately 180 amino acids, eliminating the NHR3 and NHR4 domains[84]. Using the point of truncation as a reference, the Zhang laboratory identified an alternatively spliced transcript of AML1-ETO in cells where the ETO portion of the fusion mRNA included a 155 base-pair exon (9a). The inclusion of exon 9a changes the reading frame of ETO and introduces a premature stop codon[85], thereby generating a truncated AML1-ETO protein that lacks the NHR3 and NHR4 domains. The AML1-ETO9a (AE9a) transcript is only ~20 base pairs longer than the truncated AML1-ETO cDNA that was found in the one mouse that developed AML. When cells are co-transduced with AE9a and AML1-ETO, leukemia occurs more rapidly than when AE9a is expressed alone. The identification of this AML1-ETO isoform was made more relevant by its detection in t(8;21)+ AML patients; in one report, 27 of 37 patient samples were found to contain AE9a RNA[86]. The presence of AE9a RNA appears to correlate with the presence of *c-KIT* mutations and a worse overall survival[87]. However, studies of AML1-ETO protein expression in AML have not identified a C-

terminally truncated protein of the correct size; the overwhelmingly detectable isoform of AML1-ETO is the full-length 94 kDa protein. At present, our understanding of why this truncated form of AML1-ETO induces leukemia, while its full length counterpart does not, is minimal.

Therapeutic Approaches for t(8;21) Positive AML

Treatment for t(8;21)+ AML patients typically begins with induction chemotherapy, which for decades has been the combination of cytarabine for seven days, together with three daily doses of an anthracycline (daunorubicin or idarubicin). If a complete remission is achieved, consolidation chemotherapy is given, which for t(8;21)+ AML consists of multiple courses of high dose cytarabine[88,89]. Although AML patients harboring the t(8;21) translocation are generally given a good prognosis and the majority achieve complete remission, the 5-year survival is only ~50%, and the presence of a c-kit mutation decreases the prognosis significantly[27]. Several avenues of treatment are currently being explored, and they include targeting aberrant microRNA and gene expression, as well as repurposing inhibitors for use in t(8;21)+ AML.

MicroRNAs are now recognized as fundamental components of gene regulation[90], contributing to the coordinate regulation of many developmental programs via effects on mRNA degradation and translation[91,92,93]. Given the variety of effects that AML1-ETO has on differentiation, self-renewal, and apoptosis, it is not surprising that several hematopoiesis-related microRNAs have been found to be direct AML1-ETO targets. The Nervi and Beghini laboratories have shown that miR-223 and miR-222/221 are downregulated in t(8;21)+ leukemic blasts, compared to normal bone marrow cells[94,95]. These microRNAs are expressed at low levels in primitive hematopoietic

cells, and they increase in abundance with differentiation along the granulocytic lineage[96]. By binding to an AML1 consensus binding site located 5′ of the pre-miR-223 gene, AML1-ETO silences miR-223 expression and impairs differentiation. Treating AML1-ETO positive SKNO-1 cells with the DNA methyltransferase inhibitor 5-azacytidine, or knocking down AML1-ETO expression with shRNA restores miR-223 expression and induces granulocytic maturation[94].

miR-222/221 is also directly repressed by the binding of AML1-ETO to an upstream AML1 consensus binding site[95]. Interestingly, while miR223 regulates differentiation via interactions with C/EBPα or NFI-A[96], miR-222/221 appears to regulate differentiation by targeting the 3′ untranslated region (UTR) of c-KIT mRNA[97]. The downregulation of miR-222/221 by AML1-ETO correlates with heightened c-KIT expression, providing a mechanism for the overexpression of wild type c-KIT in t(8;21)+ leukemia.

A mechanistic link between t(8;21)+ AML and mitogen-activated protein kinase (MAPK) signaling is provided by miR-24 overexpression. While miR-223 and miR-222/221 are repressed by AML1-ETO, AML1-ETO appears to activate miR-24 expression[98]. miR-24 targets the 3′ UTR of MKP-7 (a.k.a. dual-specificity phosphatase 16), lowering its expression and thereby promoting MAPK signaling via decreased dephosphorylation[99]. When miR-24 expression increases, proliferation is accelerated and granulocytic differentiation is blocked, which can contribute to the t(8;21) leukemic phenotype.

Other microRNAs may play a role in t(8;21)+ acute leukemia without being direct transcriptional targets of AML1-ETO. For instance, in a genome-wide, miRNA

expression analysis of 47 primary AML samples, miR-126 was found to be specifically overexpressed in the 10 t(8;21)+ AML samples, possibly as a result of decreased methylation within a CpG island that controls its expression. Forced expression of miR-126 in mouse bone marrow cells leads to increased proliferation and enhanced serial replating ability; these effects are amplified by the co-expression of AML1-ETO[100]. Determining whether the deregulation of miR-126, or other microRNAs, is a cause or consequence of AML1-ETO positive leukemias should help determine whether targeting specific microRNAs would be useful therapeutically.

Just as microRNAs are deregulated in t(8;21)+ AML, so has the deregulation of gene expression become a hallmark of the disease. In fact, unsupervised clustering of microarray data has allowed for the identification of leukemia subgroups, such as t(8;21)+ AML and CBFβ rearranged [inv(16) and t(16;16)] AML, based solely upon distinct gene expression signatures[101,102]. Many investigators have focused on the mechanisms underlying the repression of AML1-ETO target genes, however, many AML1-ETO target genes are upregulated following expression of AML1-ETO in U937 cells and human CD34+ cells. The relative importance of upregulated versus downregulated genes in the pathogenesis of AML1-ETO driven AML remains to be determined because these genes have generally been evaluated one at a time, an approach that is slow and painstaking. One gene, the groucho-related N-terminal enhancer of split (AES), has been identified as being consistently upregulated in t(8;21)+ AML[103,104]. Its functional importance was shown when AES knock-down in a t(8;21)+ cell line, Kasumi-1, and in AML1-ETO transduced mouse bone marrow cells decreased colony formation and methylcellulose replating ability. The self-renewal promoting effects of AES may reflect increased WNT signaling, which would be consistent with the upregulation of survivin that has also been found in t(8;21)+

AML patient samples[105]. Survivin is an inhibitor of apoptosis and a target of canonical WNT signaling; its expression is directly induced by the binding of AML1-ETO to the survivin promoter. Similar to the effects of AES knock-down, knock-down of survivin impairs the growth of AML1-ETO expressing cells, and promotes their granulocytic differentiation[106]. The therapeutic targeting of survivin is being explored in a variety of cancers[107,108] and hopefully one day will be explored in t(8;21)+ AML.

Like WNT signaling, RAS signaling has also been implicated in promoting AML1-ETO driven acute leukemia. Ras overexpression, or mutations in N-RAS that lead to its constitutive activation, are among the most frequent abnormalities found in t(8;21)+ AML[109,110], and the transduction of activated N-RAS G12D into AML1-ETO expressing human CD34+ cells results in their transformation to cytokine independence with increased colony formation and replating ability. These cells also engraft better than AML1-ETO singly expressing cells in immunodeficient mice [both NOD/SCID/IL2r$\gamma^{-/-}$ (NSG) mice and NOD/SCID mice transgenic for stem cell factor, granulocyte-macrophage colony-stimulating factor, and interleukin-3 (NSS)]. However, mice that are xenotransplanted with these CD34+ cells do not develop acute leukemia[111].

The BCL2 promoter is transcriptionally activated by AML1-ETO in leukemia cell lines[112]. This activation may be a signature of more fully transformed cells because while BCL2 is overexpressed in AML1-ETO and N-RAS G12D coexpressing CD34+ cells, it is not overexpressed in AML1-ETO singly expressing CD34+ cells. BCL2 is potentially a clinically relevant target for the treatment of AML1-ETO and N-RAS G12D coexpressing CD34+ cells, as ABT-737, an inhibitor of BCL-2 family

members, decreased their in vitro growth[111]. Though co-expression of BCL-2 with AML1-ETO does not recapitulate all of the leukemic features of N-RAS G12D and AML1-ETO co-expression, this sensitivity to ABT-737 suggests that BCL2 activation may play a role in the progressive transformation of AML1-ETO expressing cells, and could be an important therapeutic target for t(8;21)+ AML.

Other BCL-2 family members, in particular BCL-XL, may also be clinically relevant to t(8;21)+ AML. The expression of AML1-ETO in CD34+ cells activates signaling through the thrombopoietin (THPO)/MPL pathway, which upregulates BCL-XL expression. Knockdown of BCL-XL in these cells induced apoptosis, decreased in vitro colony formation, and impaired engraftment in NSG mice, while the overexpression of BCL-XL in these cells enhanced their serial replating ability and LTC-IC frequency. MPL/BCL-XL signaling appears to be upregulated in t(8;21)+ AML patient samples compared to leukemic blasts isolated from AML patients with normal cytogenetics. Targeting the THPO/MPL/BCL-XL signaling axis may be another viable therapeutic option for t(8;21)+ AML patients, as withdrawal of THPO from AML1-ETO expressing CD34+ cultures decreased BCL-XL expression, impaired cell growth, and increased apoptosis. Similar effects were not seen after withdrawal of stem cell factor (SCF) or Fms-like tyrosine kinase 3 ligand (FLT3L)[113].

Chemical inhibitors targeting t(8;21)+ AML have frequently been directed against those key proteins or pathways that are required for AML1-ETO function (Table 2). One protein, calpain B, was identified based on an RNA-interference (RNAi) screen in Drosophila and shown to be required for the effects of AML1-ETO on Drosophila blood cell numbers and differentiation capability. Encouragingly, treatment of Kasumi-1 cells with calpain inhibitors, such as N-acetyl-leucyl-leucyl-

Table 2. Inhibitors with Therapeutic Potential for t(8;21)+ AML

Inhibitor	Target	Target cell	Phenotype
ABT-737	Bcl-2 family members	AML1-ETO and N-Ras^{G12D} coexpressing CD34 $^{+}$ cells	- Decreased cell growth
ALLN and calpain inhibitor III	Calpains	Kasumi-1 cells	- Decreased viability - Decreased colony formation - Degraded endogenous AML1-ETO protein
TSA	Class I and II HDACs	Kasumi-1 cells	- Reduced the half-life of AML1-ETO protein - Induced apoptosis
DEP	Class I HDACs	Kasumi-1 cells t(8;21) AML patient bone marrow cells	- Reduced the half-life of AML1-ETO protein - Induced apoptosis - Released AML1-ETO from HSP90 - Upregulated ANXA1
17-AAG	HSP-90	Kasumi-1 cells t(8;21) AML patient bone marrow cells	- Reduced the half-life of AML1-ETO protein
SAHA	Class I and II HDACs	Kasumi-1 cells	- Induced apoptosis - Upregulated ANXA1
Methyl-prednisolone	Corticosteroid targets	Kasumi-1 cells	- Reduced colony formation ability - Induced myeloid differentiation - Induced apoptosis - Synergized with ARA-C and daunorubicin
Methotrexate	Dihydrofolate reductase	Kasumi-1 cells NOD/SCID transplanted with SKNO-1 cells	- Reduced colony formation ability - Induced myeloid differentiation - Induced apoptosis - Reduced tumor burden
NS-398	COX-2	AML1-ETO transgenic Zebrafish embryos K562 cells transduced with AML1-ETO	- Restored GATA1 expression - Removed the block in erythroid differentiation
Eri-B	Unknown	AML1-ETO positive cell lines t(8;21) AML patient bone marrow cells C57 mice transplanted with cells expressing truncated AML1-ETO Nude mice inoculated with Kasumi-1	- Induced apoptosis - Increased median survival - Reduced tumor volume of nude mice
Triptolide	Unknown	AML1-ETO positive cell lines t(8;21) AML patient bone marrow cells	- Inhibited growth and proliferation
Bortezomib	Proteosome inhibitor	AML1-ETO positive cell lines t(8;21) AML patient bone marrow cells C57 mice transplanted with cells expressing truncated AML1-ETO	- Induced apoptosis - Inhibited growth and proliferation - Increased median survival
Lys-CoA-Tat and C646	p300	AML1-ETO positive cell lines t(8;21) AML patient bone marrow cells Leukemic AML1-ETO9a positive mouse cells	- Inhibited growth and proliferation - Increased median survival of secondary transplant recipients

norleucinal (ALLN) and calpain inhibitor III, led to the degradation of AML1-ETO and a decrease in cell viability. In contrast, Calpain inhibitors had no effect on HL-60 cells or pediatric acute lymphoblastic leukemia patient samples[114]. Calpains represent a large family of Ca^{2+}-dependent cysteine proteases; however, their role in hematopoiesis is not well understood.

In a high throughput screen, Kasumi-1 cells were treated with a total of 2,480 different FDA-approved drug compounds, with promising compounds being identified as "hits" if they could recapitulate the 28 gene-member expression profile indicating "AML1-ETO abrogation." These hits fell primarily within two drug classes: corticosteroids and dihydrofolate reductase (DHFR) inhibitors, with methylprednisolone and methotrexate chosen to represent each class. Treatment with either drug reduced colony formation by Kasumi-1 cells, and induced myeloid differentiation and apoptosis. Methylprednisolone has been shown to specifically trigger apoptosis in AML1-ETO harboring cell lines, and to synergize with the traditional AML therapies, cytosine arabinoside (Ara-C) and daunorubicin[115]. Interestingly, a t(8;21)+ AML patient who received a short course of high-dose methylprednisolone for pneumonia had clearance of AML1-ETO positive leukemia cells[116]. Methotrexate appears to be less specific for AML1-ETO positive cells, and it did not synergize with Ara-C and daunorubicin in vitro. However, it did reduce leukemia cell burden in NOG mice transplanted with t(8;21)+ SKNO-1 cells[115].

A separate screen for chemical inhibitors of AML1-ETO function utilized transgenic zebra fish embryos heterozygous for AML1-ETO, which display hematopoietic features reminiscent of human t(8;21)+ AML [i.e. downregulation of GATA1 and SCL, and upregulation of myeloperoxidase (MPO)]. Transgenic embryos were incubated with 2,000 bioactive compounds, and the compounds were flagged as hits if at least 4 of the 5 embryos stained strongly for GATA1 expression. Of the 2,000 tested compounds, both nimesulide and dicumarol were able to restore GATA1 expression without altering AML1-ETO expression. Nimesulide is a known cyclooxygenase-2 (COX-2) inhibitor, and treatment of transgenic embryos with a similar inhibitor, NS-398, also triggered GATA1 upregulation. Investigations into the

COX-2 pathway revealed that AML1-ETO upregulates COX-2 expression, and may require it and prostaglandin E2 (PGE2), a cyclooxygenase metabolite, for its effects on hematopoietic cells. PGE2 has been shown to promote the proliferation of various tumor cells by activating β-catenin-dependent signaling. Indeed, knockdown of either β-catenin-1 or β-catenin-2 in transgenic embryos was able to restore GATA1 expression. Consistent with this effect, AML1-ETO significantly increased the expression of COX-2 in human K562 cells, and treatment with NS-398 removed the AML1-ETO driven block in erythroid differentiation[117]. The importance of COX/β-catenin signaling remains to be investigated in AML1-ETO mouse models and in primary cells isolated from t(8;21)+ AML patients.

In contrast to performing large scale screens to identify novel AML1-ETO inhibitors, some investigators have focused on using traditional Chinese herbs to treat t(8;21)+ AML[118], such as Eriocalyxin B (EriB), an entkaurene diterpene compound isolated from an herb of the Labiatae family, *Isodon eriocalyx* var. *laxiflora*. EriB treatment preferentially triggers the apoptosis of AML1-ETO expressing cells, possibly through the deregulation of MAPK signaling. EriB showed efficacy on five t(8;21)+ AML patients and two different mouse models of t(8;21)+ AML: C57 mice transplanted with cells expressing the C-terminally truncated AML1-ETO showed increased survival if treated with EriB (32 days for EriB at 2.5 mg/kg, compared to 25 days for control treated mice). EriB also reduced the size of subcutaneous Kasumi-1 cell tumors in a xenograft nude mouse model[119]. How EriB preferentially exerts its anti-leukemic abilities on AML1-ETO positive leukemia cells is unknown.

Another compound, Triptolide, purified from the Chinese herb Tripterygium wilfordii, also has anti-proliferative effects on SKNO-1, Kasumi-1, and t(8;21)+ AML patient cells, without significantly affecting normal blood cell growth. Triptolide may act by reducing the levels of c-KIT and NF-κB related p65, and by impairing JAK/STAT signaling[120]. These pathways are activated in AML1-ETO positive leukemia cells[121], which may explain the antileukemic effects of Triptolide.

Bortezomib, a proteasome inhibitor, appears to target leukemia cells with activated c-KIT. It blocked the proliferation and induced the apoptosis of Kasumi-1 and SKNO-1 cells, as well as CD34+ cells isolated from t(8;21)+ AML patients. Exposure to bortezomib triggered the internalization and lysosomal degradation of c-KIT. Both wildtype c-KIT and the constitutively active c-KIT mutant, N822K, phosphorylate HSP90β, which enhances the binding of HSP90β to apoptotic protease activating factor 1 (APAF-1). By inducing the degradation of c-KIT, Bortezomib indirectly decreased HSP90β phosphorylation, triggering the release of APAF-1 which leads to activation of caspase 3. Interestingly, caspase 3 is capable of cleaving AML1-ETO and AML1-ETO9a at D188, generating cleavage fragments that are deleterious to the AML1-ETO expressing Kasumi-1 cells, resulting in impaired proliferation, increased apoptosis, and decreased colony formation ability in methylcellulose. Bortezomib treatment increased the overall survival of mice transplanted with AML1-ETO9a expressing cells (34 days for Bortezomib at 2 mg/kg, compared to 19 days for control treated mice). The anti-leukemic effects of Bortezomib may relate to its ability to downregulate c-KIT and to generate AML1-ETO cleavage fragments[122].
Another study suggests that Bortezomib exerts its antileukemic effects by repressing the transcription factor specificity protein 1 (SP1)[123]. Using a ChIP-chip analysis

of AML1-ETO expressing CD34+ cells, the Alvarez laboratory determined that the majority of AML1-ETO target genes did not contain an AML1 consensus binding site in their regulatory regions, but instead showed enrichment for Sp1 consensus binding sites. Many of these genes are part of the Wnt/β-catenin pathway and the canonical stem cell pluripotency pathway, according to Ingenuity Pathways Analysis. AML1-ETO can directly bind SP1, and mithramycin A, an Sp1 inhibitor, can induce the re-expression of AML1-ETO targets that possess an Sp1 consensus binding site (but that lack an AML1 consensus binding site)[124]. By repressing SP1, Bortezomib may similarly alleviate AML1- ETO target gene repression. Regardless of the potential mechanism, Bortezomib is being evaluated in the treatment of AML.

The ability of AML1-ETO to recruit HDAC complexes to many of its target genes has prompted the testing of HDAC inhibitors on t(8;21)+ AML cells. Trichostatin A (TSA) is a hydroxamic acid-based inhibitor of class I and II HDACs[125]; it can alleviate AML1-ETO-mediated repression of AML1 target genes in reporter assays[51] and lead to the rapid degradation of AML1-ETO in Kasumi-1 cells. Similar results have been seen with Depsipeptide/romidepsin (DEP), another HDAC inhibitor that triggers the proteasomal degradation of AML1-ETO in Kasumi-1 cells[126,127,128]. Because oncogenes like AML1-ETO are frequently bound to cellular chaperones like HSP90, the Hiebert laboratory investigated the effects of the HSP90 inhibitor 17-allylamino-geldanamycin (17-AAG) on Kasumi-1 cells and found that 17-AAG also triggered the degradation of AML1-ETO. This effect was not enhanced by co-treatment with DEP, suggesting that both inhibitors act on the same pathway; indeed, subsequent investigations demonstrated that treatment with DEP releases AML1-ETO from HSP90, thereby facilitating its degradation[127]. The ability of HDAC inhibitors to alleviate AML1-ETO-mediated repression and induce the degradation of AML1-ETO

protein, possibly via acetylation of HSP90[128,129], highlights their potential in treating t(8;21)+ AML.

Acetylation can be regulated by factors other than HDACs, however, and we have discovered an important role for the lysine acetyltransferase p300 in t(8;21)+ leukemia. p300 has been shown to bind to the C terminus of AML1[20] and acetylate residues K24 and K43, thereby augmenting AML1 transcriptional activity[130]. Although the C terminus of AML1 is lost in the AML1-ETO fusion, we have found that AML1-ETO can still bind to p300 via the NHR1 domain of ETO. Binding of p300 to this region is required for the acetylation of AML1-ETO (and AML1-ETO9a) at the K24 and K43 residues. This acetylation is detectable in leukemia cells isolated from t(8;21)+ AML patients, and is essential for the ability of AML1-ETO to increase the self-renewal of human CD34+ cells. Acetylated AML1-ETO co-localizes with p300 at gene regulatory regions to cooperatively upregulate the expression of target genes that may be involved in self-renewal, such as *ID1*, *p21* and *EGR1*. In contrast, overexpression of an AML1-ETO acetylation mutant (AE K43R) in human or murine HSPCs had little effect on self-renewal. Compared to leukemogenic AML1-ETO9a, the AML1-ETO9a acetylation mutant (AE9a K43R) was unable to cause AML in mice, demonstrating that K43 acetylation is required for leukemogenesis in this mouse model[131]. Treatment of Kasumi-1 cells and t(8;21)+ AML patient cells with p300 inhibitors, such as Lys-CoA-TAT[132] or C646[133], resulted in growth inhibition, without effecting normal CD34+ cell growth. Similarly, treatment of AML1-ETO9a expressing mouse leukemia cells ex vivo with Lys-CoA-TAT or C646 prior to transplantation resulted in a significant increase in the median survival of recipient mice, compared to the DDDD-Tat or C37 treated controls. Although AML1-ETO can be regulated by a variety of post-translational modifications[134], it appears that site-

specific acetylation contributes significantly to its role as a leukemogenic oncogene. This work identifies p300 as a valid therapeutic target for t(8;21)+ AML.

Significant progress has been made in developing mouse models that recapitulate features of t(8;21)+ AML. And while these models have allowed investigators to explore avenues amenable to therapeutic targeting, the requirements for AML1-ETO driven leukemogenesis remain largely unknown. The two-hit hypothesis accounts for the ability of several factors to initiate leukemia in concert with AML1-ETO, but fails to account for the ability of others to initiate the same t(8;21)+ disease. Furthermore, the handful of mutations that successfully cooperate with AML1-ETO are present in very few t(8;21)+ AML patients and cannot represent the totality of mutations capable of contributing to AML1-ETO positive leukemogenesis.

VOLUME I:

ONE BY ONE COOPERATIVITY STUDIES

AML1-ETO and WT1

We have attempted to delineate the requirements for AML1-ETO driven leukemogenesis by investigating the leukemia that develops upon expression of AML1-ETO in WT1 overexpressing cells. While it has been shown that recipient mice die of an aggressive AML 1 to 4 months after transplantation with WT1 transgenic cells transduced with AML1-ETO[72], such a model employs artificially high levels of AML1-ETO expression not reflective of t(8;21)+ cells. An experimental setting in which WT1 is overexpressed in AML1-ETO transgenic cells, cells in which AML1-ETO is expressed at endogenous levels, has not been explored. If co-expression of AML1-ETO and WT1 is capable of satisfying the two-hit hypothesis, then an identical leukemia should result after a similar latency.

To determine if a similar leukemia can be achieved under experimental settings comparable to those of t(8;21)+ AML patients, we transduced bone marrow cells from AML1-ETO transgenic mice (*AML1-ETO-Stop/+; Mx1-Cre*$^{+/-}$)[32] with WT1 and transplanted these cells into recipient mice. 1 month post-transplantation, the recipients were treated with poly(I:C) to induce expression of AML1-ETO from the AML1 promoter. Approximately 5 months post-treatment with poly(I:C), 1 of 5 recipient mice developed AML (Figure 1A). The leukemic blasts possessed an immunophenotype reminiscent of a HSPC; they stained positive for c-KIT, with a subset also staining positive for SCA-1 (Figure 1B). These blasts were capable of

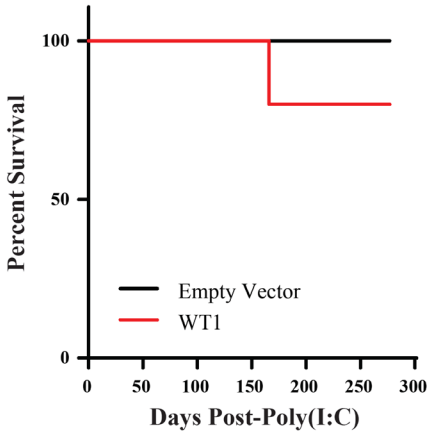

Figure 1A. Kaplan-Meier plot of mouse survival after transplantation with bone marrow cells from *AML1-ETO-Stop/+; Mx1-Cre*$^{+/-}$ mice, transduced with the given retroviral expression vector. Empty Vector (n = 5); WT1 (n = 5).

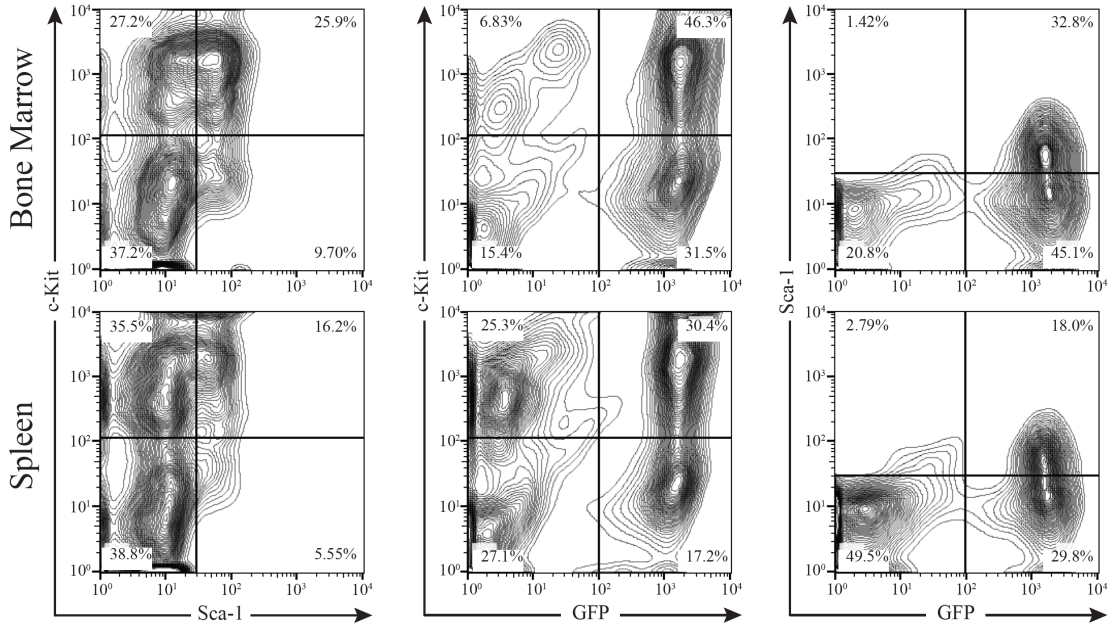

Figure 1B. Representative flow cytometry analysis of the bone marrow and spleen of the leukemic mouse transplanted with *AML1-ETO-Stop/+; Mx1-Cre*$^{+/-}$ bone marrow transduced with WT1.

transplanting the leukemia into secondary recipients (Figure 1C), with the c-KIT and SCA-1 double-positive subset becoming the dominant population. At euthanasia, both primary and the secondary recipients harbored leukemic blasts in the bone marrow in excess of 20% (Figure 1D). Blast cells were also present in the spleen (Figure 1E).

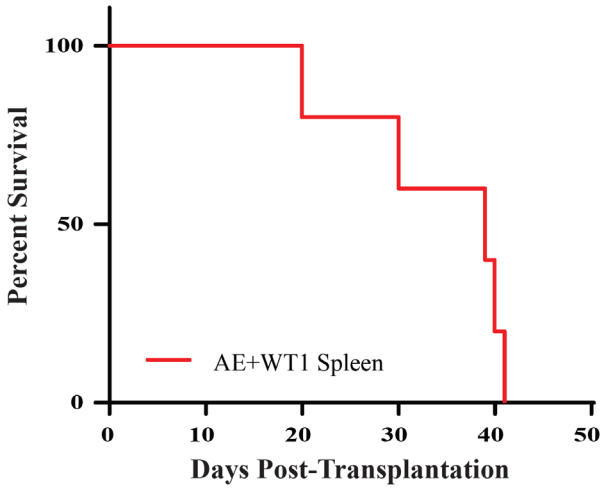

Figure 1C. Kaplan-Meier plot of mouse survival after transplantation with $2x10^6$ spleen cells from the leukemic animal of indicated primary transplantation group (n = 5).

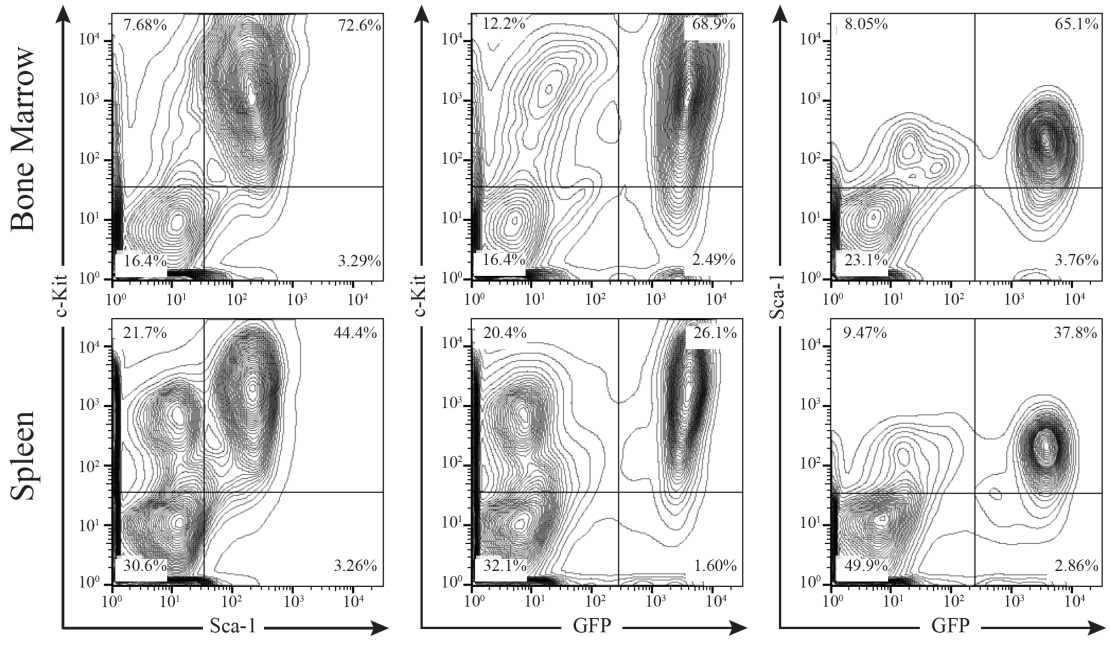

Figure 1D. Representative flow cytometry analysis of the bone marrow and spleen of secondary transplant recipients at euthanasia.

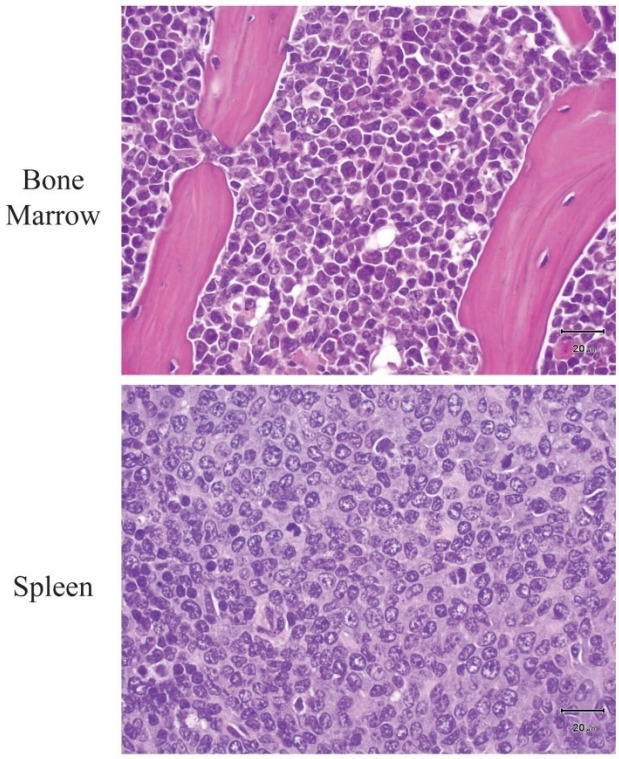

Figure 1E. Representative hematoxylin and eosin staining of the bone marrow and spleen of the leukemic mouse transplanted with *AML1-ETO-Stop/+; Mx1-Cre*$^{+/-}$ bone marrow transduced with WT1.

Although leukemia penetrance in this experimental setting was low as compared to the setting in which WT1 transgenic cells are transduced with AML1-ETO, a leukemia with similar features was capable of being induced. Expression of AML1-ETO at endogenous levels does not preclude leukemogenesis. While WT1 is not a traditional Class I mutation, its upregulation is an event that, when paired with AML1-ETO, satisfies the basic principles of the two-hit hypothesis.

AML1-ETO and PTEN Loss

A secondary event that more closely resembles a Class I mutation, and which may be capable of cooperating with AML1-ETO *in vivo*, is PTEN loss. PTEN disruption is

not a hallmark of t(8;21)+ AML; however, AML1-ETO has been found to localize at the AML1 binding site present in the PTEN promoter and downregulate PTEN expression[135]. Furthermore, the constitutive signaling through AKT that PTEN disruption engenders[136] has been documented in Kasumi-1 cells[137] and in cells isolated from the leukemia initiated by AML1-ETO and MPL co-expression[138]. Of note, both Kasumi-1 cells and the aforementioned leukemic blasts are hypersensitive to AKT inhibition[139,138].

To determine whether constitutive signaling through AKT could contribute to AML1-ETO leukemogenesis, we chose to express AML1-ETO endogenously in PTEN deleted cells by crossing AML1-ETO transgenic mice[32] (*AML1-ETO-Stop/+; Mx1-Cre*$^{+/-}$, hereafter *AE*) to PTEN conditional knockout mice[140] (*Pten*$^{fl/fl}$; *Mx1-Cre*$^{+/-}$, hereafter *PTEN*). Bone marrow cells from the aforementioned mice, as well as from the resultant progeny (*AML1-ETO-Stop/+; Pten*$^{fl/fl}$; *Mx1-Cre*$^{+/-}$, hereafter *AE / PTEN*) were then transplanted into lethally irradiated recipient mice.

Previous studies have demonstrated that 5 days after poly(I:C) injection *PTEN* mice develop a myeloproliferative neoplasm which subsequently progresses to frank leukemia. In contrast, recipients of *PTEN* cells that are treated with Poly(I:C) 6 weeks post-transplantation remain healthy; the PTEN deleted cells initially possess a proliferative advantage over co-transplanted wild type (WT) cells, but exhaust and deplete over time[140].

We similarly find that the majority of *PTEN* recipient mice remain healthy, with a select few developing T-cell lymphoma. *AE* recipient mice develop leukemia/lymphoma after a median latency of 485 days, while *AE / PTEN* recipients

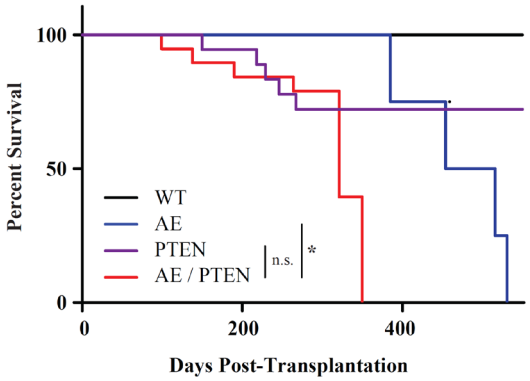

Figure 2A. Kaplan-Meier plot of mouse survival after transplantation with bone marrow cells from WT, *AE*, *PTEN*, or *AE / PTEN* mice. WT (n = 14); *AE* (n = 5); *PTEN* (n = 18); *AE / PTEN* (n = 19).

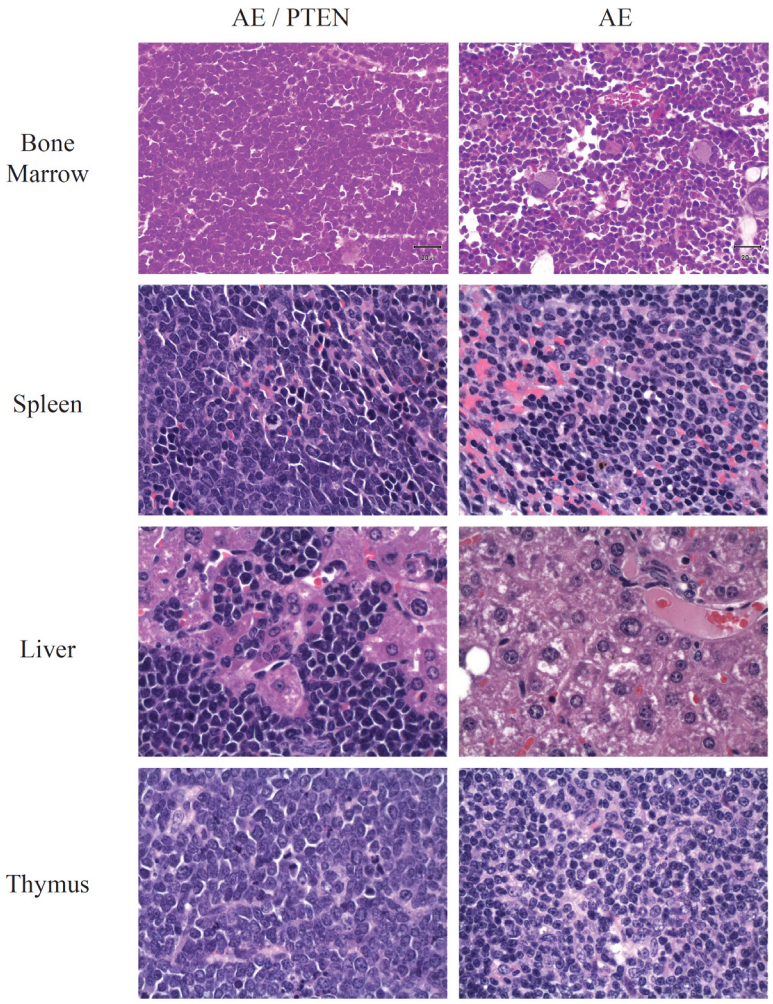

Figure 2B. Representative hematoxylin and eosin staining of the indicated organs of an *AE / PTEN* mouse with leukemia/lymphoma and a healthy *AE* mouse.

develop leukemia/lymphoma after a significantly accelerated latency of 321 days (Figure 2A). The immunophenotype of the *AE / PTEN* disease is more reflective of that found in *AE* recipients than of the T-cell lymphoma present in a subset of *PTEN* recipients. These cells, which occupy the bone marrow, spleen, liver, and thymus of recipients (Figure 2B), stain negative for the immature cell marker c-KIT. Instead, they are positive for SCA-1 and both mature cell markers CD3E and MAC-1 (Figure 2C). While the presence of MAC-1 on cells otherwise lymphoid in their surface marker expression is abnormal, it may reflect the dysregulation of surface protein genes. Notably, blast cells in a select number of *AE / PTEN* recipients are positive for phosphorylated AKT (Figure 2D), suggesting that PTEN deletion in these cells amplifies AKT signaling to a level not detectable in *AE* blasts.

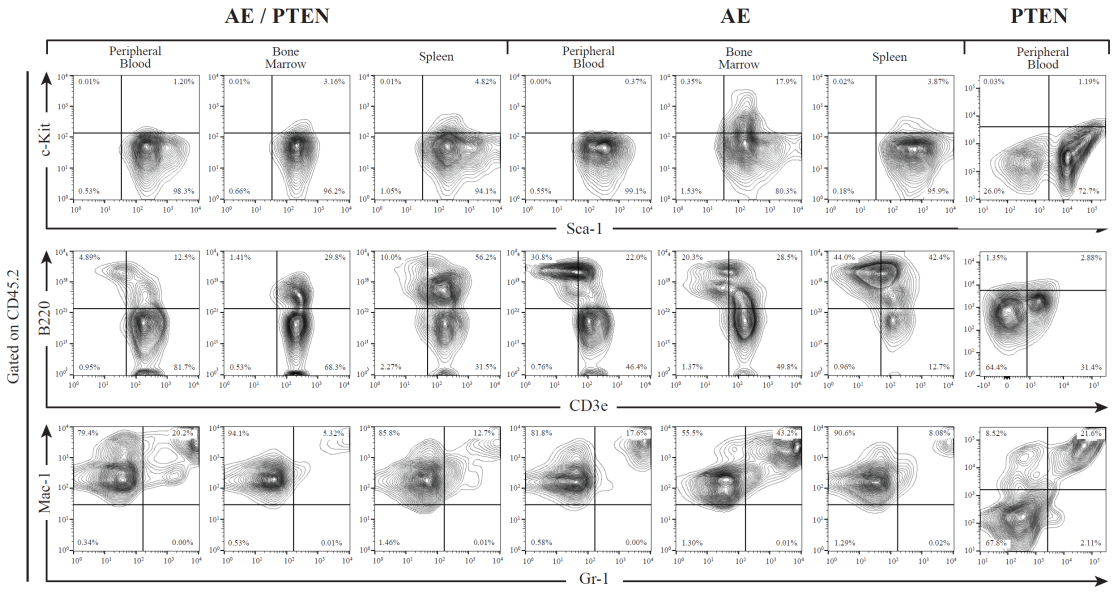

Figure 2C. Representative flow cytometry analysis of the peripheral blood, bone marrow, and spleen of an *AE / PTEN* mouse with leukemia/lymphoma, as well as healthy *AE* and *PTEN* mice.

While this model of t(8;21)+ leukemia demonstrates that PTEN loss can synergize with AML1-ETO to accelerate *AE* recipient disease, it also fails to accurately

reproduce the phenotype of AML. PTEN loss has been found to promote aneuploidy[140], which could result in *AE / PTEN* cells harboring mutations beyond enhanced AKT signaling. An alternative approach to investigating cooperativity between AML1-ETO and AKT signaling would employ a constitutively activated form of the protein, myristoylated AKT1[141]. Perhaps under this experimental setting, the affects of a single event would allow for an AML1-ETO positive disease to develop that more closely resembles that of t(8;21)+ AML patients.

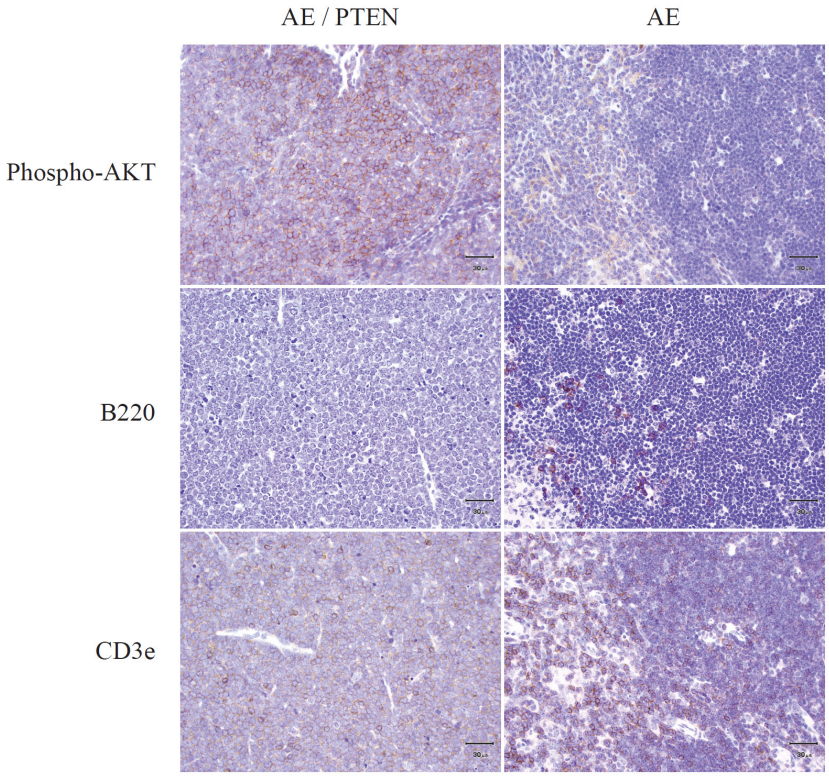

Figure 2D. Immunohistochemistry for phosphorylated-AKT, B220, and CD3e performed on the thymus of an *AE / PTEN* and an *AE* mouse with leukemia/lymphoma.

Ultimately, investigations into the requirements for AML1-ETO driven leukemogenesis will be slow and cumbersome if they depend upon cherry-picking secondary events one at a time. Instead, the field would benefit from a method that

identifies secondary events capable of cooperating with AML1-ETO in a more rapid and unbiased manner. To this end, we have developed an approach that integrates both mouse and human AML genomics. We then validated this approach by demonstrating that two of the three candidates identified by our method are capable of cooperating with AML1-ETO to develop an AML reflective of that in t(8;21)+ patients *in vivo*.

VOLUME II:

INTEGRATED APPROACH IDENTIFIES COOPERATING EVENTS*

To facilitate the discovery of cooperating mutations in t(8;21)+ AML, and develop mouse models capable of revealing new mechanisms of leukemogenesis and new therapeutic avenues, we chose to investigate two AML1-ETO driven models of AML that may depend upon the acquisition of secondary events for leukemogenesis. Expression of AML1-ETO in *p21* null cells or expression of AML1-ETO9a in WT cells, can induce fully penetrant AML after a prolonged latency[75,86]. It is thought that loss of p21 *in vivo* prevents the repair of damaged DNA and allows for the accumulation of mutations and cooperating alleles in AML1-ETO+ hematopoietic progenitors[142,143,144,145,146,147]. Studies of AML1-ETO9a driven leukemia have found deregulated expression of DNA repair genes[148,149,150], which might facilitate a similar acquisition of cooperating secondary events. We hypothesized that we could identify and credential disease alleles in t(8;21)+ AML through integrative genomic studies of human AML and the murine AMLs that arise from these models.

Identification of Phenotypically Similar Mouse Models of t(8;21)+ AML

We first expressed AML1-ETO or AML1-ETO9a in fetal liver cells isolated from WT or $p21^{-/-}$ mice. All transplant recipients of AML1-ETO expressing $p21^{-/-}$ cells developed lethal AML with a median latency of 201 days. Expression of AML1-ETO9a in WT or $p21^{-/-}$ cells resulted in a fully penetrant, lethal leukemia after a

* Hatlen MA, Arora K, Vacic V, Grabowska EA, Liao W, Riley-Gillis B, Oschwald DM, Wang L, Joergens JE, Shih AH, Rapaport F, Gu S, Voza F, Asai T, Neel BG, Kharas MG, Gonen M, Levine RL, and Nimer SD. Integrative Analysis of the Mutational Landscape of Mouse and Human AML Identifies Functionally Relevant Leukemia Disease Alleles. (2015).

median latency of 175 and 196 days, respectively (Figure 3A). At euthanasia, leukemic mice had anemia, thrombocytopenia, and elevated white blood cell counts (Figure 3B). We observed blast cell accumulation in the bone marrow, liver, and spleen (Figure 3C), resulting in splenomegaly (Figure 3D). The GFP+ blast cells display an immature immunophenotype; they are negative for lineage markers and SCA-1, but are c-KIT positive (Figure 1 E). Leukemia with an identical immunophenotype develops (data not shown) within secondary recipients (Figure 3F). The observations that these mouse models are phenotypically similar and develop AML with similar penetrance and latency suggest that they have acquired similar secondary events.

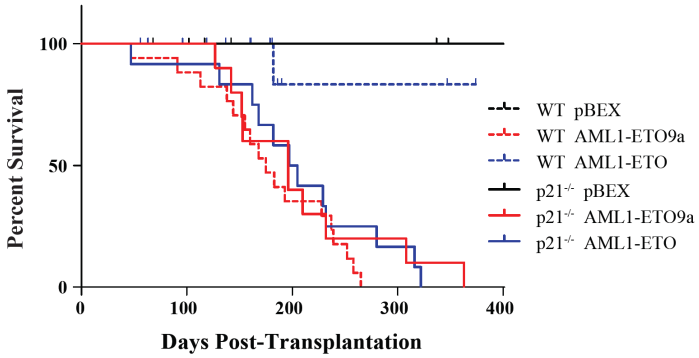

Figure 3A. Kaplan-Meier plot of mouse survival after transplantation with E14.5 fetal liver cells transduced with the given retroviral expression vector. WT pBEX (n = 12); WT AML1-ETO9a (n =17); WT AML1-ETO (n =17); $p21^{-/-}$ pBEX (n =11); $p21^{-/-}$ AML1-ETO9a (n =10); $p21^{-/-}$ AML1-ETO (n =12).

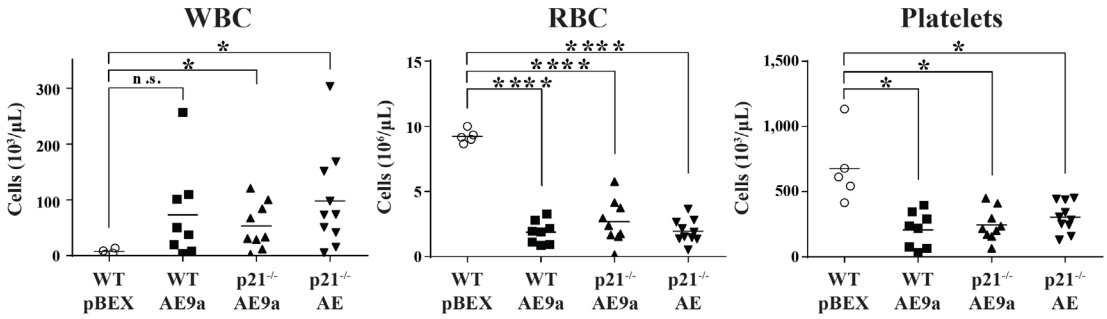

Figure 3B. Peripheral blood counts at euthanasia of leukemic mice transplanted with cells expressing AE9a or AE, as well as control mice transplanted with cells expressing pBEX. WBC indicates white blood cells; RBC, red blood cells.

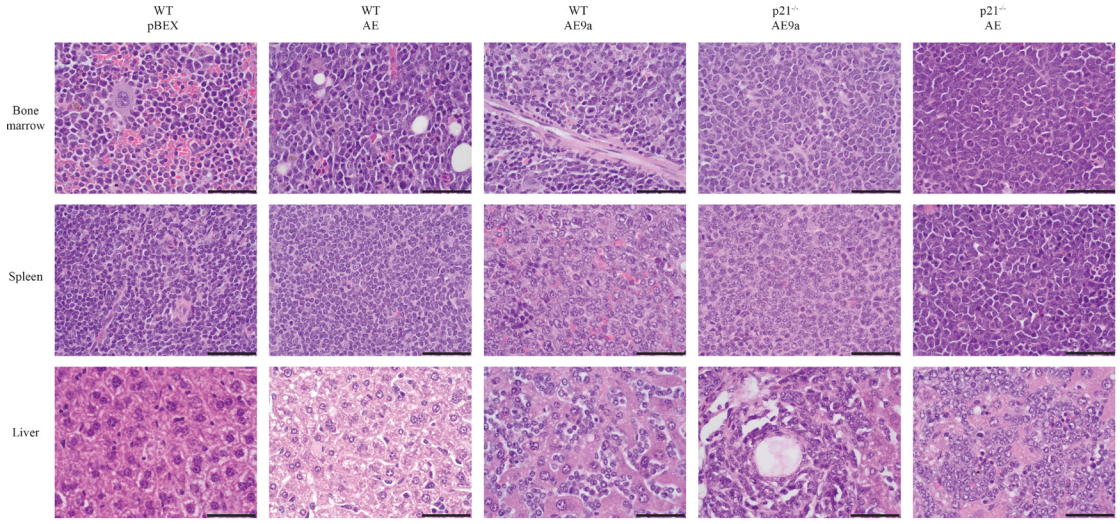

Figure 3C. Representative hematoxylin and eosin staining of the bone marrow, spleen, and liver of the following leukemic mice at euthanasia: WT AE9a, $p21^{-/-}$ AE9a, and $p21^{-/-}$ AE. Control mice, WT pBEX and WT AE, lack the accumulation of blast cells present in leukemic mice. Bar represents 50 μm.

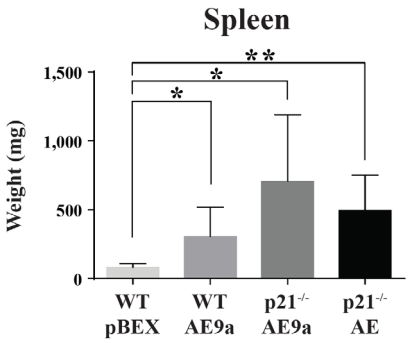

Figure 3D. At euthanasia, leukemic mice exhibit significant splenomegaly compared to control mice sacrificed at a similar time point. WT pBEX (n = 2); WT AE9a (n =8); $p21^{-/-}$ AE9a (n =5); $p21^{-/-}$ AE (n =7).

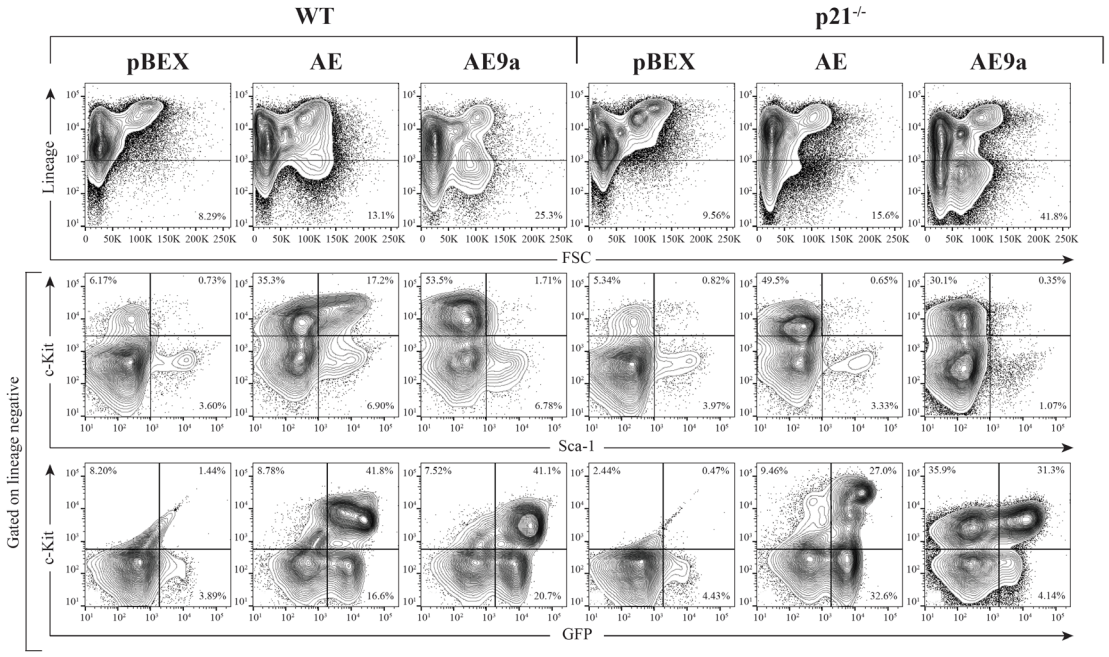

Figure 3E. Representative flow cytometry analysis of the bone marrow of mice transplanted with WT or *p21*$^{-/-}$ cells transduced with the given retroviral expression vector. Cells were analyzed for the expression of a variety of lineage markers (Lin), as well as for c-Kit, Sca-1, and GFP.

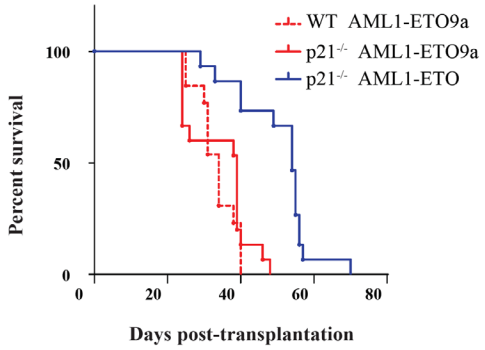

Figure 3F. Kaplan-Meier plot of mouse survival after transplantation with 2x10^{6} spleen cells from leukemic mice of the indicated primary transplantation group. WT AE9a (n =15); *p21*$^{-/-}$ AE9a (n =13); *p21*$^{-/-}$ AE (n =15).

Mouse Models of AML Reproduce the Genomic Landscape of Human AML

We next investigated the mutational landscape of these murine AMLs and compared it to a somatic mutational analysis of other murine AML models and AML patients[151]. Sequence analysis revealed that a majority of murine AML samples were highly concordant with pooled control samples, allowing us to exclude germline variants. We did not observe a significant difference in the number of variants detected or genes targeted in the mouse models compared to AML patient samples (Figure 4A-C). When we restricted our analysis to genes for which human-mouse orthology is known, we observed a significant enrichment in the murine AML for genes (hypergeometric $p \leq 4.26x10\text{-}20$) (Figure 4D) and protein domains (hypergeometric $p \leq 4.23x10\text{-}3$) mutated in human AML. Domains mutated in both human and murine AML were similarly affected by recurrent mutations (Spearman correlation of domain p-values $r = 0.53$, $p \leq 2.73x10\text{-}8$) (Figure 4E). We next investigated the frequency of mutations in different protein classes, and while the protein classes targeted in murine vs. human AML were significantly different ($p = 0.049$), we found no differences in the protein classes targeted in AML1-ETO/AML1-ETO9a murine AML vs. human t(8;21)+ AML ($p = 0.327$) (Figure 4F).

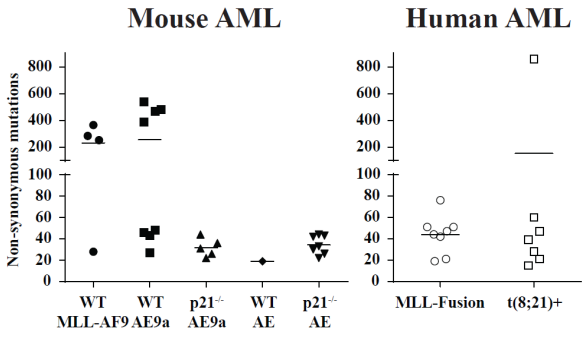

Figure 4A. The number of non-synonymous mutations in the GFP+ spleen cells of leukemic mice and in human AML patients with the given cytogenetic abnormality.

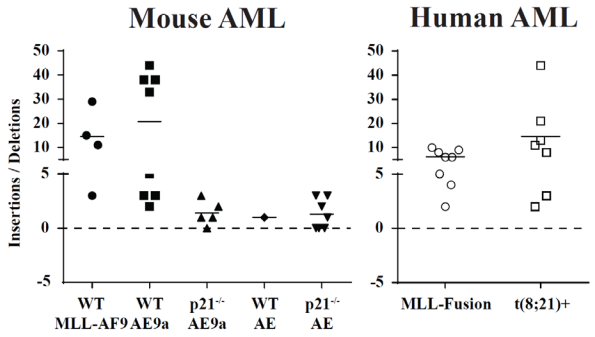

Figure 4B. The number of insertion and deletion events in the GFP+ spleen cells of leukemic mice and in human AML patients with the given cytogenetic abnormality.

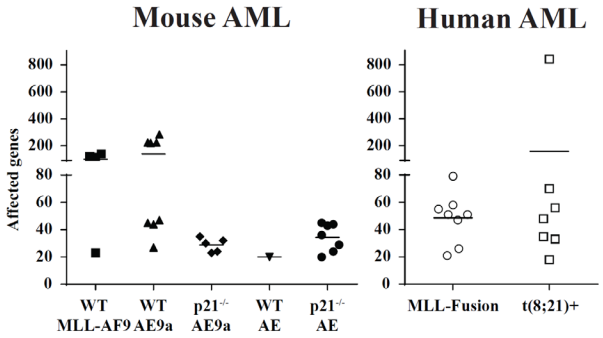

Figure 4C. The number of genes harboring non-synonymous, insertion, or deletion events in the GFP+ spleen cells of leukemic mice and in human AML patients with the given cytogenetic abnormality.

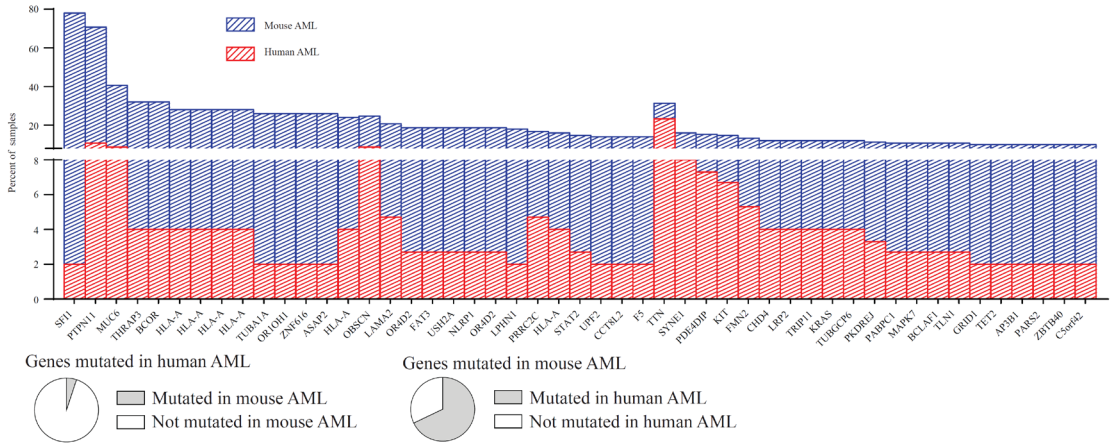

Figure 4D. A stacked plot of the genes that are mutated in ≥ 2% of human AML samples and ≥ 8% of mouse AML samples. 67.90% of genes mutated in leukemic mice are also mutated in human AML patients, while only 5.07% of genes with a protein-coding mutation in AML patients are also mutated in leukemic mice. This enrichment is significant ($p \leq 4.26 \times 10^{-20}$).

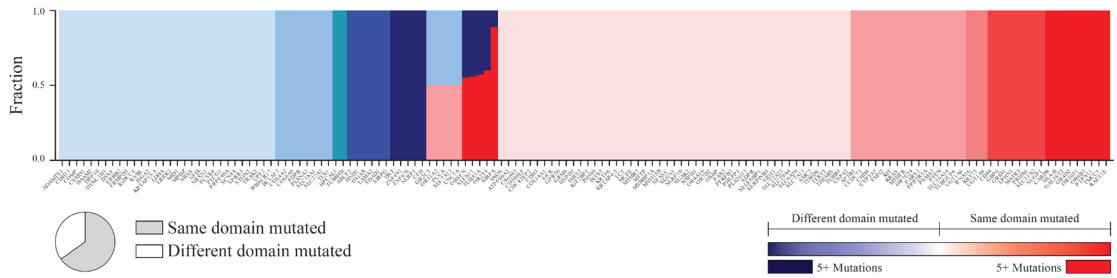

Figure 4E. Of the genes that harbor protein-coding mutations in both leukemic mice and human AML patients, the fraction of protein domains that are mutated in both species (red) or in only one species (blue) is depicted. The color intensity increases with the number of mutations in a given protein domain. 65.07% of these protein domains are mutated in both species, demonstrating enrichment ($p \leq 4.23 \times 10^{-3}$). Domains similarly affected are recurrently mutated (Spearman correlation of domain p-values, $r = 0.53$, $p \leq 2.73 \times 10^{-8}$)

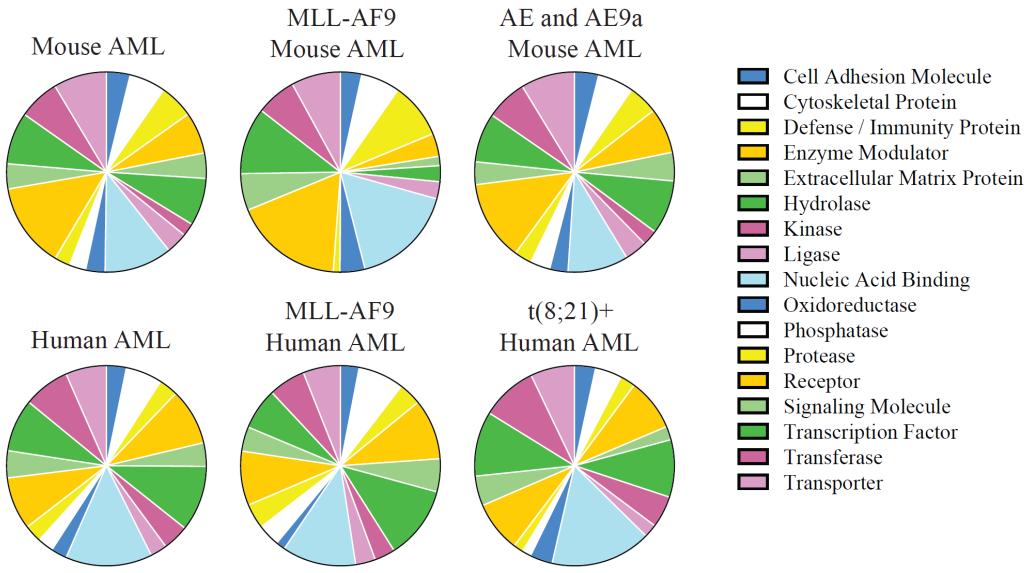

Figure 4F. Genes mutated in mouse and human AML belong to the given protein classes. There is a significant difference in the classes that are targeted between mouse and human AML ($p = 0.049$), but not between t(8;21)+ mouse and human AML ($p = 0.327$).

Integrated Genomic Approach Identifies Potential Cooperating Events in t(8;21)+ AML

We next sought to mine the mutational data to identify mutations capable of cooperating with AML1-ETO, initially focusing our efforts on the 424 genes mutated in both human and murine AML, and then further narrowing our focus to those genes that were also significantly mutated (defined as Genome MuSiC SMG FDR ≤ 30%). This reduced the number of potential candidates from 424 to 38 human genes, representing 45 mouse orthologues (Figure 5A). We focused on the mouse orthologues which were significantly mutated (FDR ≤ 10%) in AML1-ETO driven leukemias, and not in MLL-AF9 driven leukemia, and identified 38 mouse genes which met this criteria (Figure 5B). These 38 genes correspond to 32 human orthologues, which include 3 alleles annotated in COSMIC as cancer genes[152]: TET2, PTPN11, and THRAP3.

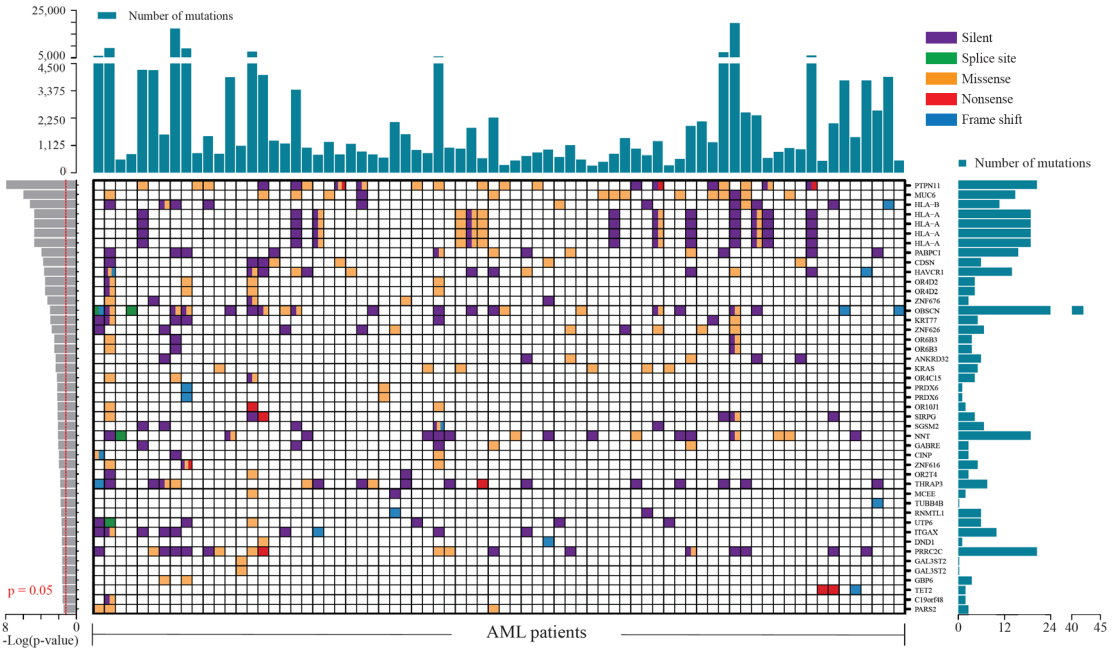

Figure 5A. The patients that harbor non-synonymous mutations in genes that are significantly mutated in mouse and human AML (FDR ≤ 30%).

Figure 5B. The types of mutations present in mouse AML samples that occur in genes significantly mutated in mouse and human AML. Those genes that are significantly mutated in a given mouse AML group (FDR ≤ 10%) are highlighted in blue under the given group.

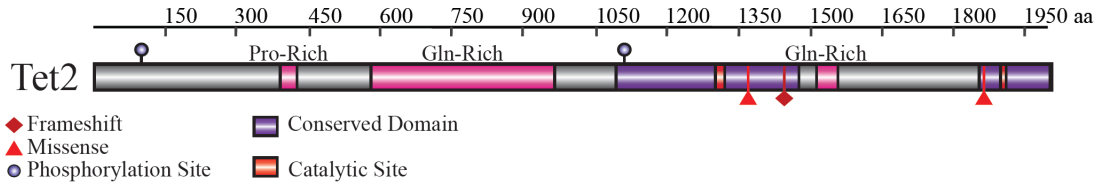

Figure 5C. A schematic of TET2 and the location of mutations identified in t(8;21)+ AML patients.

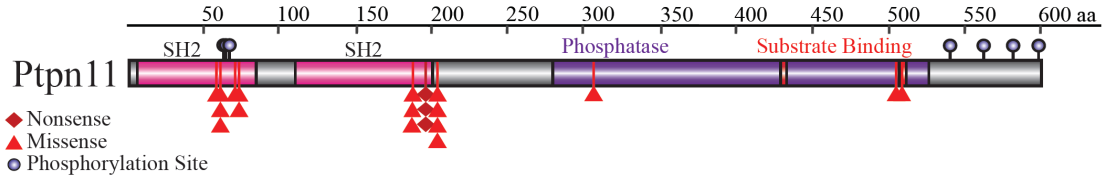

Figure 5D. A schematic of PTPN11 and the location of mutations identified in AML patients.

Expression of AML1-ETO in Tet2 Null Cells Generates a Novel Model of t(8;21)+ AML

The variants detected in TET2 in AML patients include nonsense, frameshift, and missense mutations within conserved domains, consistent with loss of TET2 enzymatic function (Table 3). Notably, 3 of the 29 t(8;21)+ AML patients enrolled in the ECOG E1900 trial[153] harbored TET2 mutations (Figure 5C). In vivo, monoallelic or biallelic loss of *Tet2* in the hematopoietic compartment of mice results in myeloproliferation and extramedulary hematopoiesis, but not AML[154,155].

To determine if loss of *Tet2* can cooperate with AML1-ETO in vivo, we transduced bone marrow cells from *Vav-Cre*$^{+}$*Tet2*$^{-/-}$ (*Tet2* KO) mice with AML1-ETO or AML1-ETO9a and transplanted the cells into lethally irradiated recipients. All mice transplanted with AML1-ETO expressing *Tet2* KO cells developed fully penetrant leukemia, while mice transplanted with AML1-ETO9a expressing *Tet2* KO cells

Table 3. The *TET2* Mutations in AML Patients

TET2 Mutations	Effect	% of *TET2* Mutations	Patient Database
Q481	Nonsense	50.00%	TCGA
R550	Nonsense	50.00%	
270	Frameshift	1.96%	ECOG E1900
S327X	Missense	1.96%	
P426L	Missense	1.96%	
K433X	Missense	1.96%	
E452A	Missense	1.96%	
R544X	Missense	1.96%	
R550X	Missense	1.96%	
586	Frameshift	1.96%	
Q622X	Missense	1.96%	
F868L	Missense	1.96%	
Q891X	Missense	1.96%	
912	Frameshift	1.96%	
Q916X	Missense	1.96%	
921	Frameshift	1.96%	
958	Frameshift	1.96%	
966	Frameshift	1.96%	
W1003X	Missense	1.96%	
Q1021R	Missense	1.96%	
1034	Frameshift	1.96%	
Q1084P	Missense	1.96%	
1114	Frameshift	1.96%	
1118	Frameshift	1.96%	
E1141K	Missense	1.96%	
H1219Y	Missense	1.96%	
N1260K	Missense	1.96%	
R1261C	Missense	5.88%	
G1283D	Missense	1.96%	
W1292R	Missense	1.96%	
1299	Frameshift	1.96%	
1322	Frameshift	1.96%	
R1365H	Missense	1.96%	
G1369V	Frameshift	1.96%	
1395	Frameshift	1.96%	
E1405X	Missense	1.96%	
1439	Frameshift	1.96%	
1448	Frameshift	1.96%	
S1486X	Missense	1.96%	
Q1524X	Missense	1.96%	
R1572W	Missense	1.96%	
H1817N	Missense	1.96%	
E1851K	Missense	1.96%	
I1873T	Missense	3.92%	
1893	Frameshift	1.96%	
R1896M	Missense	1.96%	
S1898F	Missense	1.96%	
Y1902X	Missense	1.96%	
1960	Frameshift	1.96%	
P1962L	Missense	1.96%	

developed a significantly accelerated leukemia with a median latency of 118 days ($p < 0.0001$) (Figure 6A). At euthanasia, leukemic recipients had anemia, thrombocytopenia, elevated white blood cell counts (Figure 6B), and blast cell infiltration into the liver and spleen, with consequent splenomegaly (Figure 6C-D).

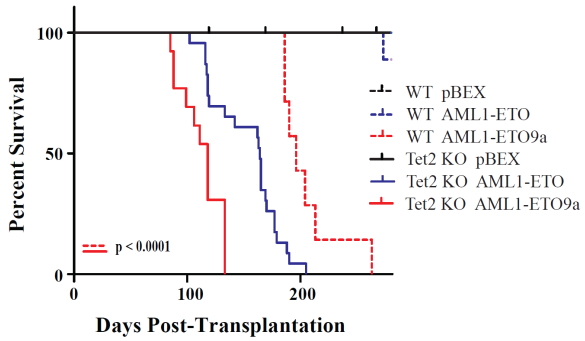

Figure 6A. Kaplan-Meier plot of mouse survival after transplantation with bone marrow cells transduced with the given retroviral expression vector. WT pBEX (n =5); WT AE9a (n =7); WT AE (n =10); Tet2 KO pBEX (n =12); Tet2 KO AE9a (n =13); Tet2 KO AE (n =23).

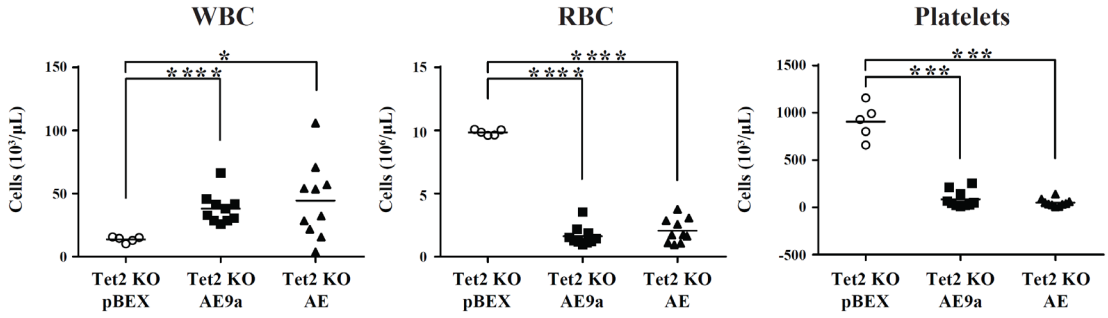

Figure 6B. Peripheral blood counts of mice transplanted with Tet2 KO cells expressing AE9a, AE, or pBEX.

The GFP+ blast cells are lineage negative, SCA-1 positive or negative, and c-KIT positive (Figure 6E). The observed cooperativity between *Tet2* loss and AML1-ETO in vivo implicates TET2 loss-of-function mutations in t(8;21)+ AML patients as functional cooperating events.

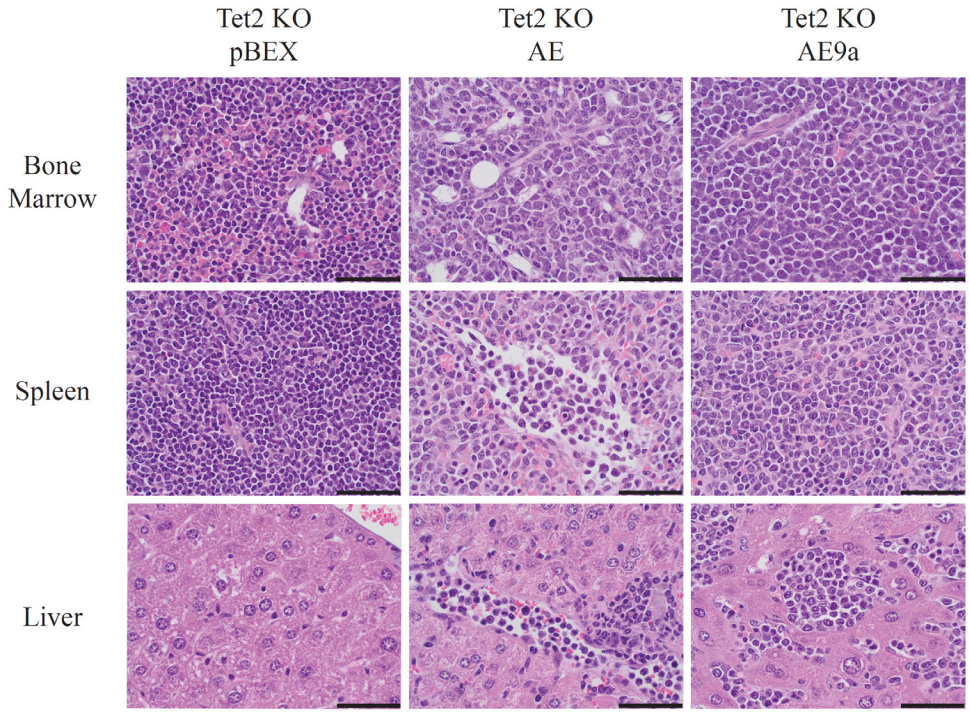

Figure 6C. Representative hematoxylin and eosin staining of the bone marrow, spleen, and liver of leukemic mice transplanted with Tet2 KO AE or AE9a cells. Tet2 KO pBEX recipients lack the accumulation of blast cells present in leukemic mice. Bar represents 50 μm.

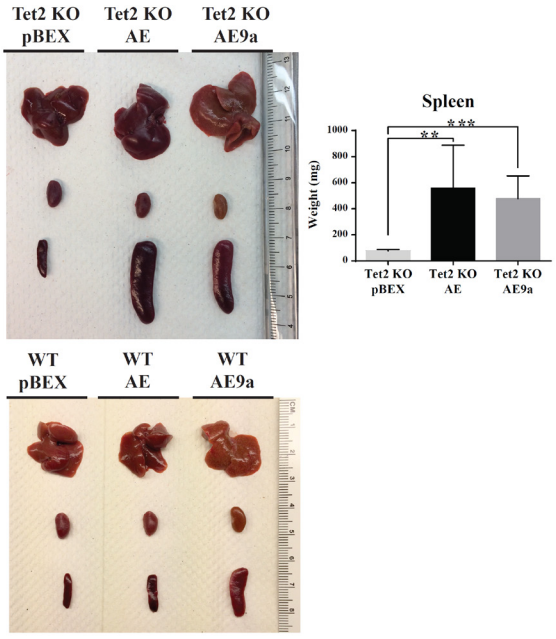

Figure 6D. At euthanasia, leukemic mice exhibit significant splenomegaly compared to control mice sacrificed at a similar time point. Tet2 KO pBEX (n =2); Tet2 KO AE (n =9); Tet2 KO AE9a (n =9). From top to bottom: Liver, kidney, spleen.

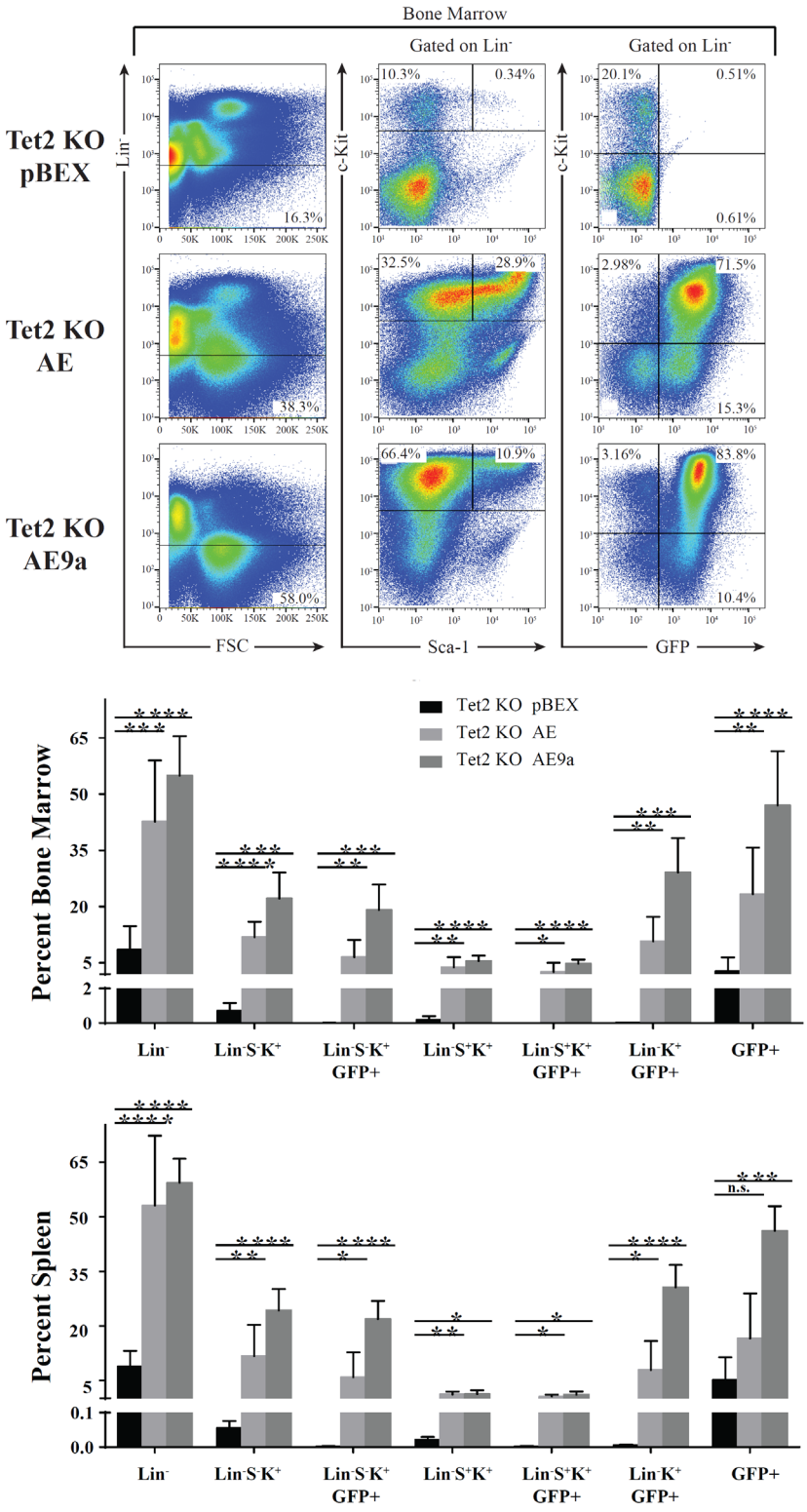

Figure 6E. Flow cytometry analysis of the bone marrow and spleen of mice transplanted with Tet2 KO cells transduced with the given retroviral expression vector. Lin indicates lineage panel; S indicates Sca-1; K indicates c-Kit.

Expression of AML1-ETO with PTPN11 D61Y Generates a Novel Model of t(8;21)+ AML

The PTPN11 mutations that we observe in human and mouse AML occur within highly conserved regions of PTPN11; the SHP2 domains and the domain required for PTPN11 phosphatase activity (PTP) (Figure 5D, Table 4). Because PTPN11 is auto-

Table 4. The *PTPN11* Mutations in AML Patients

PTPN11 Mutations	Effect	% of *PTPN11* Mutations	Patient Database
G60V	Missense	5.00%	TCGA
D61Y	Missense	5.00%	
D61V	Missense	10.00%	
F71L	Missense	5.00%	
T73I	Missense	10.00%	
S189A	Missense	15.00%	
Y197*	Nonsense	15.00%	
L206L	Missense	20.00%	
N308D	Missense	5.00%	
S502P	Missense	5.00%	
Q510L	Missense	5.00%	

inhibited by interactions between N-SHP2 and PTP[156,157], mutations in these domains activate phosphatase activity, stimulating RAS signaling[158,159]. Of note, PTPN11 A72V has been reported in a t(8;21)+ AML patient[160] and activating RAS mutations occur in 8-10% of AML1-ETO+ AML patients, implicating constitutive MAPK signaling in AML1-ETO+ AML pathogenesis[60]. We previously showed that mice expressing the constitutively active D61Y mutation in the hematopoietic compartment (*Ptpn11*^{D61Y}) develop a fatal myeloproliferative neoplasm. However, bone marrow cells from these mice fail to engraft beyond 20 weeks in transplant recipients and do not induce AML in vivo[161]. We therefore transduced bone marrow cells from

Ptpn11^{D61Y} mice with AML1-ETO and transplanted the cells into lethally irradiated recipients. Mice expressing both alleles developed fatal AML (Figure 7A).

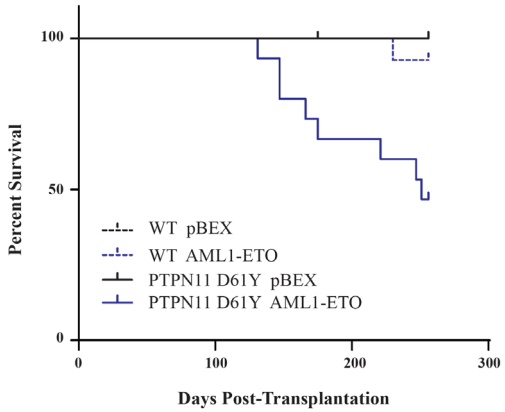

Figure 7A. Kaplan-Meier plot of mouse survival after transplantation with bone marrow cells transduced with the given retroviral expression vector. WT pBEX (n =10); WT AE (n =15); PTPN11 D61Y pBEX (n =10); PTPN11 D61Y AE (n =15).

At euthanasia, leukemic mice had elevated white blood cells counts, anemia, thrombocytopenia (Figure 7B), and blast cell accumulation in the bone marrow, liver and spleen (Figure 7C-D). These GFP+ blasts were SCA-1 negative and c-KIT positive (Figure 7E) and capable of transmitting the disease into secondary recipients (Figure 7F-H, Table 5). Thus, the mutant PTPN11 protein can cooperate with AML1-ETO to induce AML in vivo.

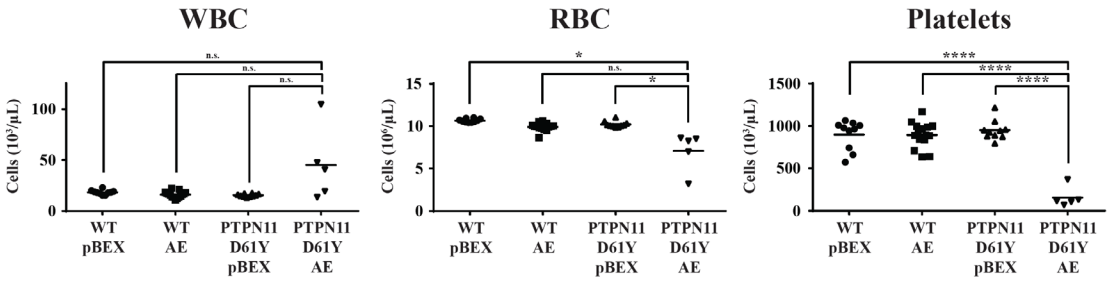

Figure 7B. Peripheral blood counts at euthanasia of leukemic PTPN11 D61Y AE mice, as well as control mice.

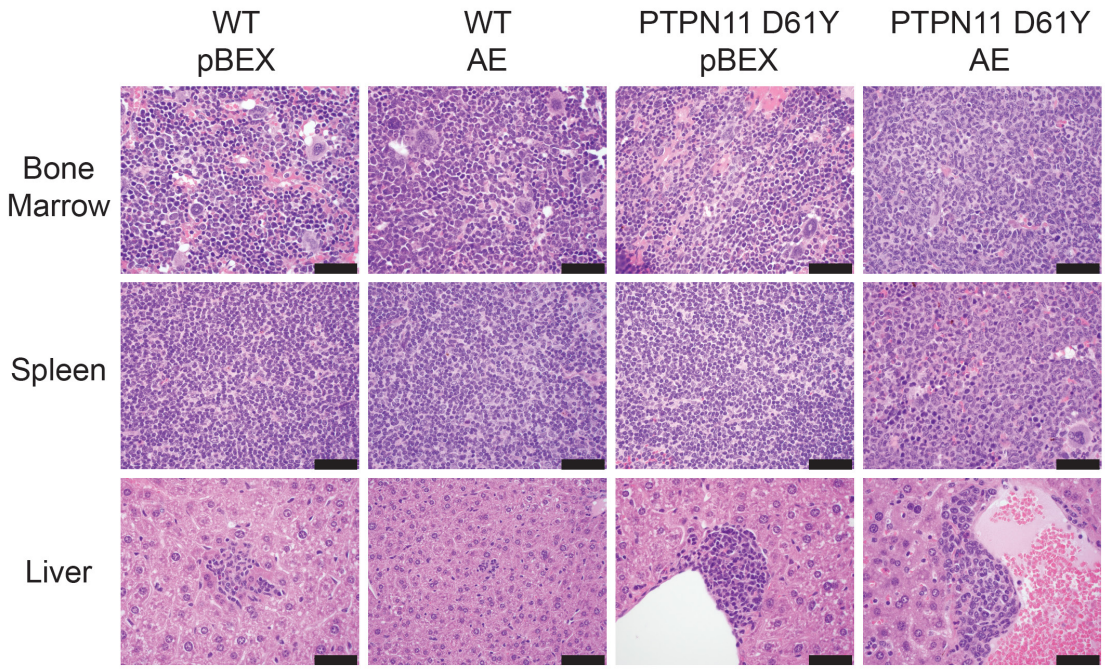

Figure 7C. Representative hematoxylin and eosin staining of the bone marrow, spleen, and liver of the given transplant recipients. Bar represents 50 μm.

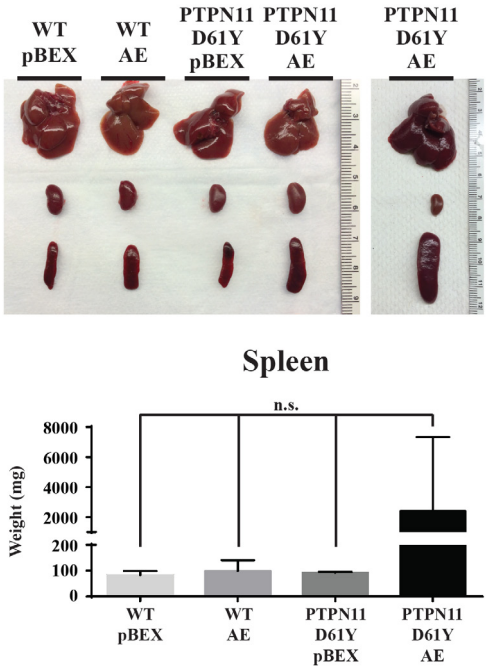

Figure 7D. At euthanasia, leukemic mice exhibit splenomegaly compared to control mice sacrificed at a similar time point. WT pBEX (n =3); WT AE (n =3); PTPN11 D61Y pBEX (n =3); PTPN11 D61Y AE (n = 6). From top to bottom: Liver, kidney, and spleen.

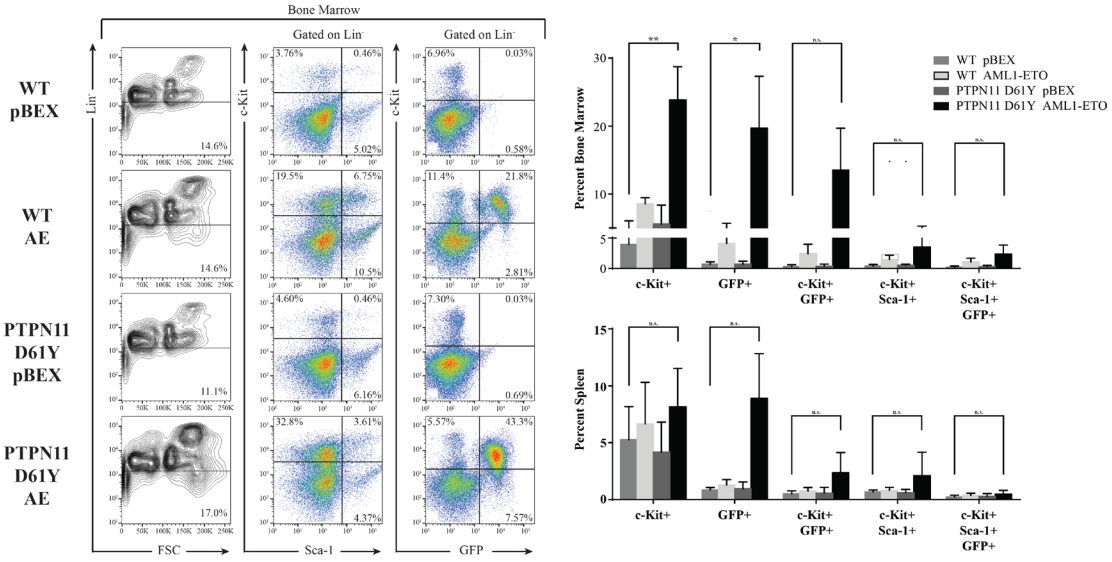

Figure 7E. Flow cytometry analysis of the bone marrow and spleen of mice transplanted with WT or PTPN11 D61Y cells transduced with the given retroviral expression vector.

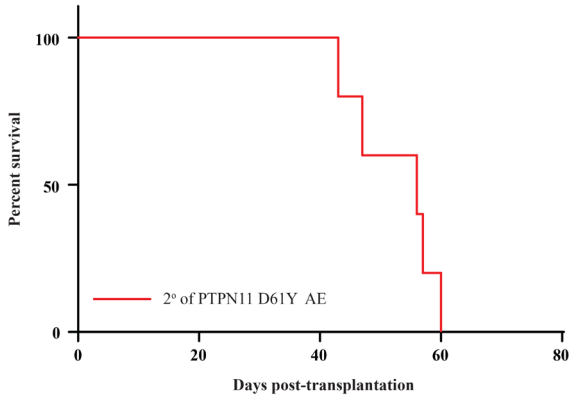

Figure 7F. Kaplan-Meier plot of mouse survival after transplantation with spleen cells from a leukemic PTPN11 D61Y AE mouse (n =5).

Figure 7G. Secondary recipients of PTPN11 D61Y AE leukemic cells develop splenomegaly. From top to bottom: Liver, kidney, and spleen.

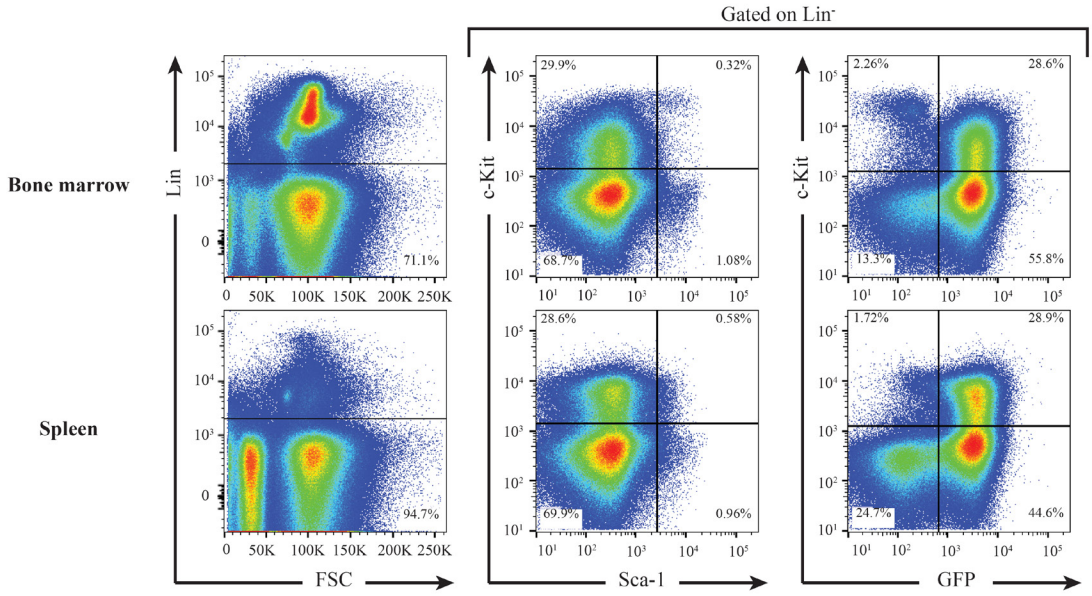

Figure 7H. Representative flow cytometry analysis of the bone marrow and spleen of mice transplanted with leukemic PTPN11 D61Y AE cells.

Table 5. Peripheral Blood Counts of Recipients of leukemic *Ptpn11*^{D61Y} AE cells

WBC (10^3 cells/μL)	RBC (10^6 cells/μL)	Platelets (10^3 cells/μL)	Spleen (g)
60.79 ± 18.56	3.00 ± 1.09	88.00 ± 44.07	1.04 ± 0.12

WBC, white blood cells; RBC, red blood cells. Values are averages ± standard deviation.

Dependence of t(8;21)+ Models on Secondary Events

Given that the mouse models generated by expressing AML1-ETO9a or AML1-ETO in transplanted p21 null cells reproduce the genetic landscape of human AML and acquire mutations capable of cooperating with AML1-ETO, we decided to investigate

these models' requirements for leukemia progression in the hopes of identifying new therapeutic targets for t(8;21)+ AML.

While lack of p21 in these leukemias is thought to promote leukemogenesis by facilitating the acquisition of secondary, cooperating events, the absence of p21 itself may be necessary for leukemia progression. To ascertain the role of p21 in these leukemias, we expressed p21 exogenously in p21 null leukemic spleen cells expressing AML1-ETO and either transplanted the cells into sub-lethally irradiated recipients or placed the cells into methylcellulose media.

Recipients of leukemic cells expressing p21 died of leukemia after a median latency of 55 days, a latency significantly longer than that of mice receiving leukemic cells null for p21 (Figure 8A).

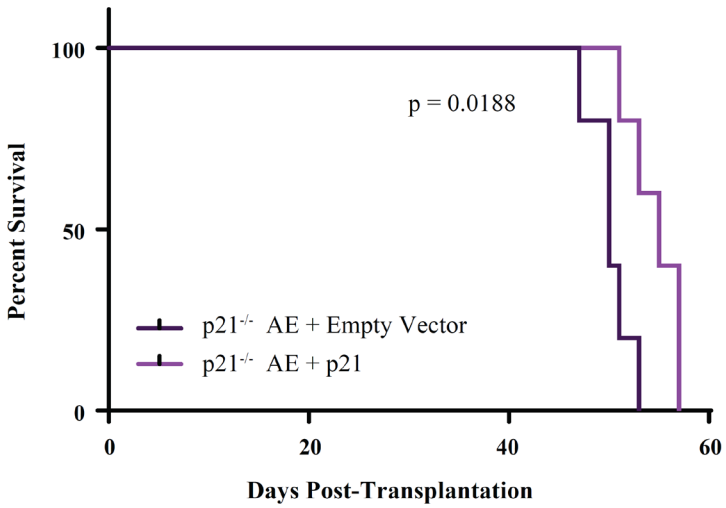

Figure 8A. Kaplan-Meier plot of mouse survival after transplantation with spleen cells from a leukemic p21 null AE mouse, which exogenously express either an empty vector or a vector containing the cDNA for p21 (n =5).

Similarly, while leukemic cells exogenously expressing p21 showed no impairment in their ability to form colonies that consistently replated in methycellulose media, these cells overall form fewer colonies than leukemic cells null for p21 (Figure 8B).

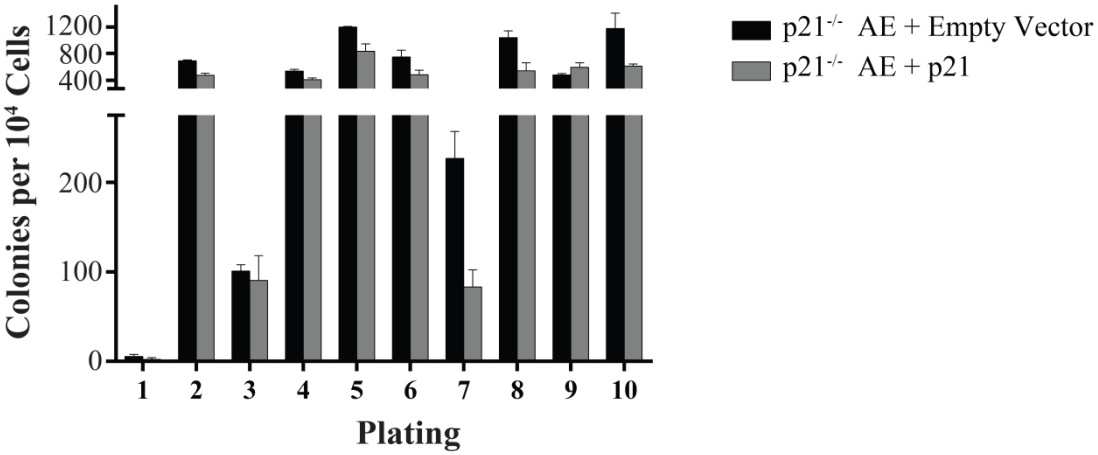

Figure 8B. The ability of leukemic spleen cells null for p21 and expressing AE to form colonies that replate in methylcellulose after an empty vector or cDNA for p21 is exogenously expressed (n=3).

To further investigate the dependence of leukemia maintenance on p21, we transplanted colonies from the fourth methylcellulose replating into sub-lethally irradiated recipients. Notably, recipients of cells expressing p21 developed leukemia after a significantly extended median latency of 66 days, compared to recipients of p21 null cells, which developed leukemia after a median latency of 33 days. When leukemias from these transplant recipients were serially transplanted, a majority of the secondary recipients of p21 expressing leukemia cells survived the entirety of the observation period without developing disease, while all secondary recipients of p21 null leukemia cells succumbed to disease after a median latency of 41 days (Figure 8C). Interestingly, at euthanasia, all recipients lacked expression of p21, regardless of

whether they received p21 null cells or p21 null cells exogenously expressing the gene (Figure 8D). This result suggests that p21 plays a role not only in the initiation of these leukemias, but in their maintenance as well.

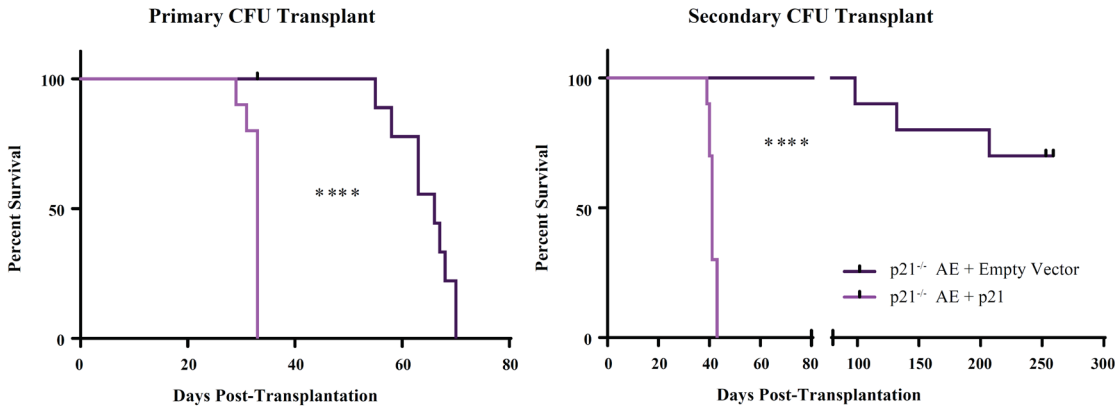

Figure 8C. Kaplan-Meier plot of mouse survival after serial transplantation with colonies of leukemic cells expressing the given vector from the fourth methylcellulose replating (n =10).

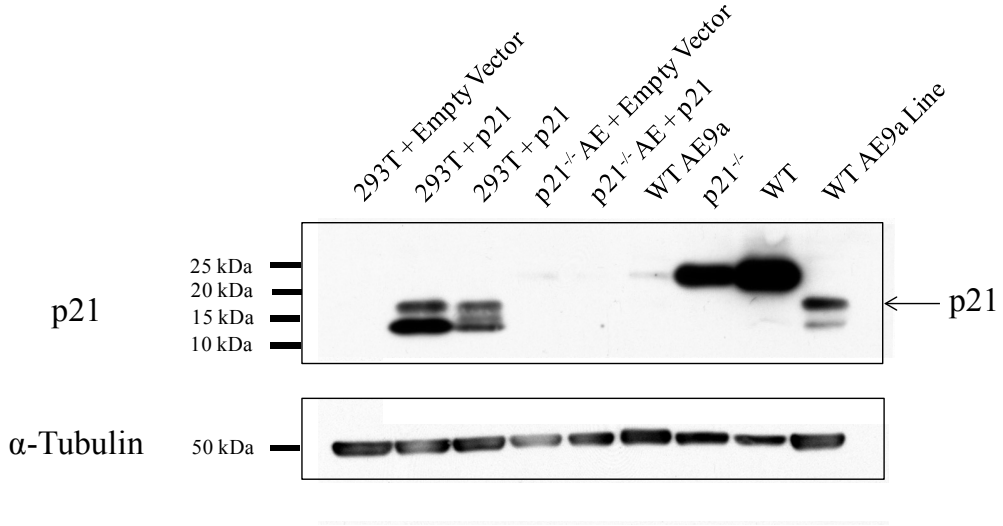

Figure 8D. The expression level of p21 in various cell lines or in primary spleen cells: 293T cells transfected with an empty vector or p21; GFP+ spleen cells from leukemic mice transplanted with p21 null AE cells exogenously expressing empty vector or a vector containing p21 cDNA; GFP+ spleen cells from a mouse transplanted with WT cells expressing AE9a; p21-/- or WT spleen cells; a cell line generated from the in vitro culture of leukemic spleen cells from a mouse transplanted with WT cells expressing AE9a.

In studying leukemias expressing AML1-ETO9a, or AML1-ETO in the p21 null setting, we were able to identify Tet2 loss as an event capable of cooperating with AML1-ETO to induce leukemia. Tet2 loss also significantly increased the latency of AML1-ETO9a leukemias. Given the role of Tet2 as a demethylating agent, we decided to investigate whether a global demethylating agent, 5-Azacytidine, could impact the maintenance of primary leukemias. When we treated mice receiving leukemic cells expressing AML1-ETO9a with 5-Azacytidine, we found that the median latency was significantly extended to 77 days. Vehicle treated mice died of leukemia after a median latency of 38 days (Figure 8E). A specific methylation profile may be necessary for the maintenance of AML1-ETO9a and AML1-ETO leukemias.

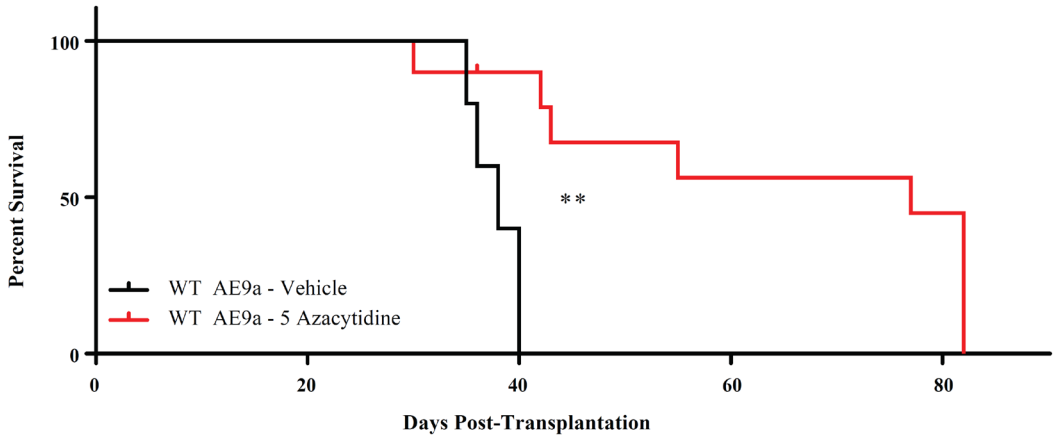

Figure 8E. Kaplan-Meier plot of mouse survival after treatment with 100ug of 5-Azacytidine. Mice were transplanted with spleen cells from a leukemic WT AE9a mouse and treatment occurred for 5 consecutive days at 30 and 60 days post-transplantation (WT AE9a - Vehicle, n =5; WT AE9a - 5 Azacytidine, n =10).

DISCUSSION*

Although the first report of t(8;21) occurred over 40 years ago, the requirements for AML1-ETO driven leukemogenesis remain largely unknown. Our own efforts to identify WT1 overexpression and PTEN loss as events capable of cooperating with endogenous AML1-ETO were not particularly successful, despite the fact that these events fulfilled tenets of the two-hit hypothesis. These two events yielded a leukemia with incomplete penetrance and a leukemia/lymphoma that did not reflect t(8;21)+ patient disease, respectively.

In an effort to more rapidly identify AML1-ETO cooperating partners and better understand the requirements for t(8;21)+ leukemogenesis, we investigated the genomics of both mouse and human AML. Exome sequencing revealed that two phenotypically similar AML1-ETO driven murine leukemia models possess a mutational landscape reflective of each other and human AML patients, especially in terms of the number of acquired secondary events, the genes involved, and the protein domains targeted. By identifying genes that were significantly mutated in AML patients and the mouse models, we were able to identify *Tet2* loss and PTPN11 D61Y as secondary events capable of cooperating with AML1-ETO in vivo.

The observation that *Tet2* loss can cooperate with AML1-ETO challenges the traditional "two-hit hypothesis" of AML, which posits that AML1-ETO commonly cooperates with mutations that activate oncogenic signaling[68,29]. Our data suggest that

* Hatlen MA, Arora K, Vacic V, Grabowska EA, Liao W, Riley-Gillis B, Oschwald DM, Wang L, Joergens JE, Shih AH, Rapaport F, Gu S, Voza F, Asai T, Neel BG, Kharas MG, Gonen M, Levine RL, and Nimer SD. Integrative Analysis of the Mutational Landscape of Mouse and Human AML Identifies Functionally Relevant Leukemia Disease Alleles. (2015).

mutations in epigenetic regulators can potently cooperate with AML1-ETO in vivo. Given that neomorphic mutations in IDH1/2 and loss-of-function mutations in WT1 also attenuate TET2 function and reduce DNA hydroxymethylation in vivo[162,163,164,165], it will be important to determine if altered DNA hydroxymethylation and consequent methylation is a common pathogenetic event in AML1-ETO+ AML. Indeed our finding that a global demethylating agent attenuates the oncogenic effects of AML1-ETO9a implicates DNA methylation as a critical component of t(8;21)+ AML.

To better define the role of methylation in t(8;21)+ AML, we believe that studies should be initiated which utilize mouse models of AML1-ETO expression and either IDH1/2 mutations, WT1 mutations, Tet2 loss, or WT1 overexpression. We have demonstrated that the latter two events are sufficient for leukemogenesis in t(8;21)+ cells despite their antithetical impact on 5hmC; WT1 overexpression is synonymous with increased global 5hmC, a feature absent from Tet2 null cells[164]. Measuring the levels of 5hmC in these AML1-ETO driven mouse models will be critical to uncovering whether loss of 5hmC is an event conserved across AML1-ETO driven models which rely upon co-occurring mutations in epigenetic regulators, or if, rather than global methylation, it is the hypermethylation of specific gene targets that is conserved across these models. WT1 is responsible for recruiting TET2 to gene targets such that their expression is activated[165]. When WT1 is overexpressed, its promiscuous activity may result in the recruitment of TET2 to novel genes at the expense of proper targets, ensuring the continued methylation and repression of these targets. Similarly, in the case of WT1 loss-of-function mutations or neomorphic IDH1/2 mutations, gene targets may fail to be demethylated because of WT1's inability to recruit TET2 or attenuated TET2 function. Gene silencing of WT1/TET2

targets, such as BTRC, DACT1, and TBL1X[165], may represent a novel mechanism of leukemogenesis in t(8;21)+ AML. For this reason, explorations into the epigenetic profile of t(8;21)+ AML with concurrent mutations in epigenetic regulators, and their potential response to epigenetic therapies, are warranted.

Additional mechanistic studies can also be performed on the primary AML1-ETO driven models of leukemogenesis, those models which reflect the mutational profile of AML patients and that rely upon the absence of p21. While we initially hypothesized that the role of p21 in these models was to facilitate the acquisition of secondary disease alleles, it has become clear that p21 loss is necessary not only for leukemia initiation but also progression. p21 plays a number of biological roles, from ensuring the repair of damaged DNA to regulating cellular self-renewal and cell cycle progression[166]. To determine which of these particular roles impacts leukemia progression, and to potentially identify new avenues of therapy for t(8;21)+ AML, it would be beneficial to serially transplant p21 null, AML1-ETO expressing leukemia cells that have been engineered to exogenously express a mutant form of p21 incapable of binding cyclin-cdk complexes and regulating cell cycle progression[167]. In this manner, we could assess whether increased cycling is necessary for leukemia maintenance and if cell cycle inhibitors should be investigated for therapeutic efficacy in t(8;21)+ AML.

The observation that AML1-ETO can cooperate with PTPN11 D61Y is more consistent with the two-hit hypothesis, and further implicates RAS signaling in t(8;21)+ AML. While KRAS is significantly mutated in the human AML panel and 20% of the AML1-ETO9a expressing *P21*$^{-/-}$ leukemias had KRAS G12C mutations, previous studies pairing AML1-ETO with activating RAS mutations in vivo have had

mixed results[111,168,169]. Our data showing that PTPN11 D61Y is capable of cooperating with AML1-ETO underscores the critical role of dysregulated RAS/MAPK signaling in AML1-ETO+ leukemogenesis and suggests that MAPK directed therapies should be investigated in this genetically defined t(8;21)+ AML subset.

Most importantly, our study illustrates the power of integrating murine and human genomic profiling to identify functionally relevant disease alleles in AML. Previous studies in murine solid tumors[170] and APL[171] have identified functional cooperating disease alleles; our data suggest that systematic comparisons of mutational patterns in murine and human malignancies is warranted as they can be used to identify cooperating disease alleles with mechanistic and therapeutic relevance.

METHODS*

Construction of Expression Vectors and Virus Production

The cDNAs encoding AML1-ETO or AML1-ETO9a were digested with NotI and cloned into the NotI multiple cloning site of the MSCV-IRES-BEX plasmid (pBEX), upstream of the IRES and blue-excited GFP motifs. pBEX differs from the previously described MigR1 plasmid[172] by featuring cDNA for enhanced GFP that has been altered such that it can be distinguished from violet-excited GFP[173]. To produce retrovirus capable of expressing AML1-ETO or AML1-ETO9a, 293T cells were cotransfected with a pBEX plasmid and MCV-Ecopac via calcium phosphate precipitation[174].

Fetal Liver and Bone Marrow Transduction and Transplantation

Fetal liver cells were harvested from E14.5 WT (Jackson Laboratory) or *P21*$^{-/-}$ (kindly provided by Tyler Jacks) C57BL/6 embryos, while bone marrow cells from adult *Vav-Cre*$^{+}$ or *Vav-Cre*$^{+}$*Tet2*$^{-/-}$ C57BL/6 mice[154] were harvested 6 days after intraperitoneal administration of 200 mg/kg of 5-fluorouracil (Sigma). Bone marrow cells were harvested from 9 week old *Mx1-Cre*$^{+/-}$ or *Ptpn11*^{D61Y} mice that had been treated at 3 weeks with poly(I:C) as previously published[161]. Harvested cells were cultured in RPMI media supplemented with 10% fetal bovine serum, 10 ng/ml IL-3, 10 ng/ml IL-6 and 100 ng/ml stem cell factor (Peprotech) prior to transplantation, with 8 ug/ml of

* Hatlen MA, Arora K, Vacic V, Grabowska EA, Liao W, Riley-Gillis B, Oschwald DM, Wang L, Joergens JE, Shih AH, Rapaport F, Gu S, Voza F, Asai T, Neel BG, Kharas MG, Gonen M, Levine RL, and Nimer SD. Integrative Analysis of the Mutational Landscape of Mouse and Human AML Identifies Functionally Relevant Leukemia Disease Alleles. (2015).

polybrene (Sigma) added during spinoculation. Transduced cells were transplanted into 8-10 week old female C57BL/6 recipient mice by tail-vein injection after the recipient mice had been lethally irradiated with 950 cGy, given in a split dose separated by 3 hours. Procedures performed on these mice were approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center.

Exome Sequencing

Genomic DNA from GFP+ mouse spleen cells or mouse tail tissue was isolated using phenol-chloroform extraction. Exome capture was performed using the SureSelect Mouse All Exon Kit (Agilent Technologies) and sequencing was performed using an Illumina platform. Approximately 80 million reads per sample were acquired after a paired-end run with 75 or 100 base-pairs. Paired-end reads were aligned to the NCBIM37.67/mm9 mouse reference genome using the Burrows-Wheeler Aligner, BWA aln v0.6.2[175] and processed using the best practices pipeline that included marking of duplicate reads by the use of Picard tools (http://picard.sourceforge.net), and realignment around indels and base recalibration via Genome Analysis Toolkit (GATK) ver. 3.1.1[176]. Somatic single nucleotide variants (SNVs) were called using muTect v1.1.4[177], Virmid v1.1.0[178] and Strelka v1.0.12[179]; and somatic indels via SomaticIndelDetector v2.3.9 and Strelka. SNVs and indels were filtered using the default filtering criteria as implemented in each of the callers and annotated via snpEff and snpSift[180,181]. In addition, all germline SNVs and indels identified in the 17 key mouse strains as part of the Mouse Genome Project[182] were filtered out of the call set. To compare our results to what has been previously discovered in human patients, we downloaded the BAM files for the 150 human AML exomes from The Cancer

Genome Atlas project[151]. For consistency, we extracted the reads from human exomes, re-processed them, and called somatic variants using the same pipeline as for mouse exomes.

SMG and Domain Analysis

Significantly mutated genes were identified in mouse and human samples using the SMG module from Genome MuSiC v0.4[183]. Homology between mouse and human genes was based on the mouse-human homolog list downloaded from The Jackson Laboratory web site (http://www.informatics.jax.org/homology.shtml). False discovery rates (FDRs) were determined using the likelihood ratio test from the Genome MuSiC SMG module. We annotated proteins with protein domains from Pfam-A[184], a database of high quality, manually curated protein families (http://pfam.xfam.org). We used the hmmscan algorithm from HMMER3.0 package to match protein sequences against the Pfam-A database.

Statistical Analysis

Statistical significance between two groups was assessed using the Student's t-test with Welch's correction; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; n.s., not significant. The hypergeometric test was used to determine if there was significant overlap in the genes that are mutated in mouse and human AML, as well as if there was significant overlap in the protein domains that are affected in genes mutated in both mouse and human AML. The significance of recurrently mutated protein domains was tested using the binomial test, where p = length of domain (in AA) / length of protein (in AA). To determine if mouse leukemias were significantly

different from human leukemias with respect to the frequency with which all protein classes were affected by gene mutations, protein classes annotated using PANTHER[185] were compared across the two groups using the Pillai-Bartlett statistic from a multivariate analysis of variance (MANOVA) model. The corresponding p-values were computed from a transformed value of the test statistic which has an approximate F distribution. All calculations were done using R version 3.0 (www.r-project.org).

Flow Cytometry

To determine the immunophenotype of normal and leukemic populations, cells were stained with a panel of antibodies against lineage markers, each of which was conjugated to APC-Cy7: CD3, CD4, CD11b, CD19, CD45R, Gr-1, NK1.1, and TER119. Cells were also stained with anti-CD117 (c-KIT) and anti-SCA-1, conjugated to PE and PE-Cy7 respectively (BioLegend). Stained cells were analyzed using an LSR Fortessa or LSR II flow cytometer (BD), and data were analyzed using FlowJo software.

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